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ISGylation and phosphorylation, two protein posttranslational modifications that play important roles in influenza A virus replication

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ISGYLATION AND PHOSPHORYLATION, TWO PROTEIN POSTTRANSLATIONAL MODIFICATIONS THAT PLAY IMPORTANT ROLES IN INFLUENZA A VIRUS REPLICATION

by

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Dedication

To

my grandpa, C.H. Hsiang

my grandma, K.C. Hsiang

my dad, Lee Hsiang

my brother, Tien-Ruey Hsiang

for their love and support.

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ISGylation and phosphorylation, two protein modifications that play important roles in influenza A virus replication.

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Two posttranslational modifications, ISGylation and phosphorylation, impact the replication of influenza A virus, a human pathogen responsible for high mortality pandemics. The ubiquitin-like ISG15 protein is induced by type 1 interferon (IFN) and is conjugated to many cellular proteins by three enzymes that are also induced by IFN. Experiments using ISG15-knockout (ISG15-/-) mice established that ISG15 and/or its conjugation inhibits the replication of influenza A virus, but inhibition was not detected in mouse embryo fibroblasts in tissue culture. The present study is focused on the effect of ISG15 and/or its conjugation on the replication of influenza A virus in human cells in tissue culture. IFN-induced antiviral activity against influenza A virus in human cells was significantly alleviated by blocking ISG15 conjugation using small interfering RNAs (siRNAs) against ISG15 conjugating enzymes. IFN-induced antiviral activity against influenza A virus gene expression and replication was reduced 10-20-fold by suppressing ISG15 conjugation. Unconjugated ISG15 does not contribute to this antiviral activity. Consequently human tissue culture cells can be used to delineate how ISG15 conjugation

inhibits influenza A virus replication. SiRNA knockdowns were also used to demonstrate that other IFN-induced proteins, specifically p56, MxA and phospholipid scramblase 1, also inhibit influenza A virus gene expression in human cells. The research on phosphorylation focused on the viral NS1A protein, a multifunctional virulence factor. Although threonine phosphorylation of the NS1A protein was discovered 30 years ago, the sites of phosphorylation and its function had not been identified. A recombinant influenza A virus encoding an epitope-tagged NS1A protein was generated, enabling the purification of NS1A protein from infected cell extracts. Mass spectrometry identified phosphorylation at T49 and T215. A recombinant virus in which phosphorylation at 215 was abolished by replacing T with A is attenuated, and an apparently aberrant NS1A protein is produced. Attenuation did not occur when T was changed to E to mimic a constitutively phosphorylated state, or surprisingly when T was changed to P to mimic avian NS1A proteins. These results suggest that T215 phosphorylation in human viruses and P215 in avian viruses can support analogous functions.

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1.1 INFLUENZA VIRUS

1.1.1 Overview

Influenza A viruses are important respiratory pathogens which cause significant mortality and morbidity throughout the world every year. Among the three major human pandemics occurred last century, the "Spanish flu" in 1918 was the most deadly one and cost more than 30 million lives. The other two, "Asian flu" in 1957 and "Hong Kong flu" in 1968, also resulted in significant mortality rate. Pandemic influenza viruses usually result from gene reassortments between different strains, a process called "antigenic shift". The reassortants encode proteins which are distinct from the previous circulating strains, and as a result, there is little immunological protection against these new viruses in human populations. The recently isolated highly virulent avian influenza H5N1/1997 and H5N1/2004 have led to the speculation of the emergence of new pandemic outbreak. Most H5N1 viral infections had been limited to avian-to-human transmissions. These infections caused severe respiratory symptoms in infected human patients and resulted in high mortality rate. If the H5N1 virus acquires the ability of efficient human-to-human transmission by gene reassortments and/or mutations, it would be expected to cause the next human pandemic and possibly claim millions of lives.

Influenza A virus is a member of the *Orthomyxoviridae* family, which also includes 3 other genera: influenza virus B, influenza virus C, and Thogotovirus, distinguished by the antigenic differences between their nucleoproteins (NP) and matrix proteins (M1). These viruses are enveloped single-stranded (-)RNA viruses with a segmented genome. Both influenza A and B viruses contain eight genomic RNA

segments, whereas influenza C contains only seven genomic RNA segments. Influenza A virus is responsible for the most severe influenza pandemics in the past and has a broad host range including humans, pigs, horses, whales, seals, and birds. Influenza B and C viruses infect mainly humans (Lamb and Krug 2001).

Influenza A viruses can be further divided into different subtypes based on the antigenic variation of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently there are 16 known subtypes of HA (H1-H16) and 9 known subtypes of NA (N1-N9). While all subtypes can be found in wild aquatic birds, since they are the natural hosts of influenza viruses, only H1N1, H2N2, H3N2, H5N1, H7N7, and H9N2 subtypes have been isolated from human. Among them, three HA subtypes, H1, H2, H3, and two NA subtypes, N1 and N2, have caused human epidemics (Julkunen et al. 2001).

1.1.2 Structure of influenza A virus

The influenza A virus contains a bilayer phospholipid envelope derived from the host cell membrane during the viral budding process (Lamb and Krug 2001). Three viral proteins, HA, NA, and M2, which are embedded in the lipid envelope, are encoded by segments 4, 6, and 7, respectively. The HA and NA proteins are anchored in the envelope by short sequences of hydrophobic amino acids and form the spike-shaped outer layer (Figure 1.1). The HA protein is a homotrimer, and is responsible for binding to the sialic acid-containing receptors on the cell surface and for subsequent membrane fusion that releases the internal components into the cell. The NA protein, which forms a homotetramer, destroys receptors by hydrolyzing sialic acid from glycoproteins and facilitates the release of viral progeny. The M2 protein is an integral membrane homotetramer, which functions as an ion channel by pumping hydrogen ions (H+) into the virus particle, which is essential for virus uncoating and the later migration of viral

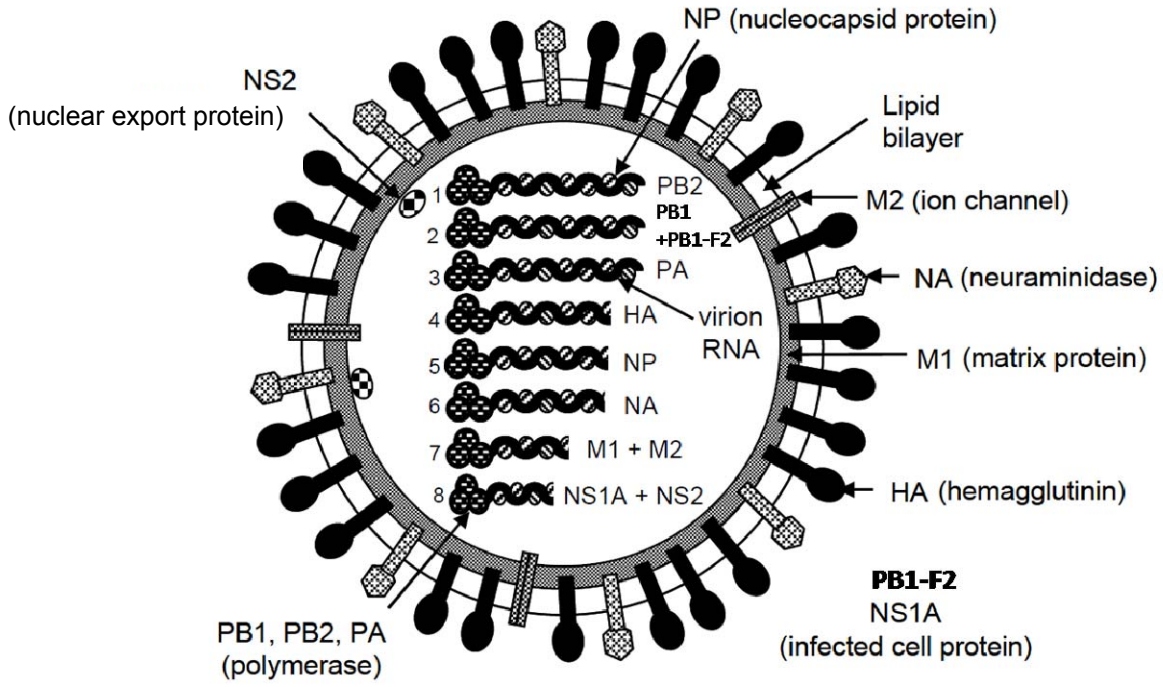


Figure 1.1 Schematic diagram of the structure of influenza A virion. The location of the structural viral proteins, viral envelope, and the eight genomic RNA segments are indicated. The NS1A and PB1-F2 are found only in infected cells, and not in virions. Modified from Noah and Krug 2005.

core ribonucleoprotein complex (RNPs) into the nucleus. Under the lipid envelope lays a layer of M1 protein, which is also encoded by segment 7 that gives rise to M2 protein as a result of alternative splicing. M1 is the most abundant protein in the viral particle with important structural roles and regulatory functions associated with RNPs. M1 mediates the encapsidation of the viral ribonucleoprotein complexes into the viral envelope. Inside the virion, all eight viral RNA segments are bound to the NP and the viral RNA polymerase to form ribonucleoprotein(RNP) complexes (Figure 1.2). The heterotrimeric RNA polymerase complex contains 3 subunits: PB2, PB1 and PA, which are encoded by the three largest vRNA segments, segment 1, 2, and 3 respectively. The NP protein, encoded by segment 5, is a non-specific single stranded RNA (ssRNA) binding protein, and each NP monomer interacts with approximately 20 nucleotide of the vRNA. Both ends of the vRNA interact with each other to form a supercoil structure, and the RNA polymerases interact with both ends of the vRNA within the virion. The three polymerases and NP proteins are the minimum combination which is required for influenza virus genome replication and RNA transcription. The NS2A protein (Mr ~14,000), encoded by segment 8 by alternative splicing with +1 frameshift of its ORF compared to NS1A, is also present in virions in low amounts associated with the M1 protein and functions as a nuclear export protein for vRNP in infected cells (NS2/NEP). The eight segments of influenza A virus genome encodes 10, or 11, proteins. Viral mRNAs from segment 1 through 6 are basically monocistronic. However, the viral mRNA encoded by segment 2 of some viruses contains a short alternative reading frame that encodes the PB1-F2 protein which has a role in apoptosis. Viral mRNAs from segments 7 and 8 undergo alternative splicing and thus encode two proteins. Among all the proteins encoded by influenza A virus genome, only the NS1 protein from segment 8, and, in some cases, the PB1-F2 protein from segment 2, are non-structural proteins.

Influenza virus RNP

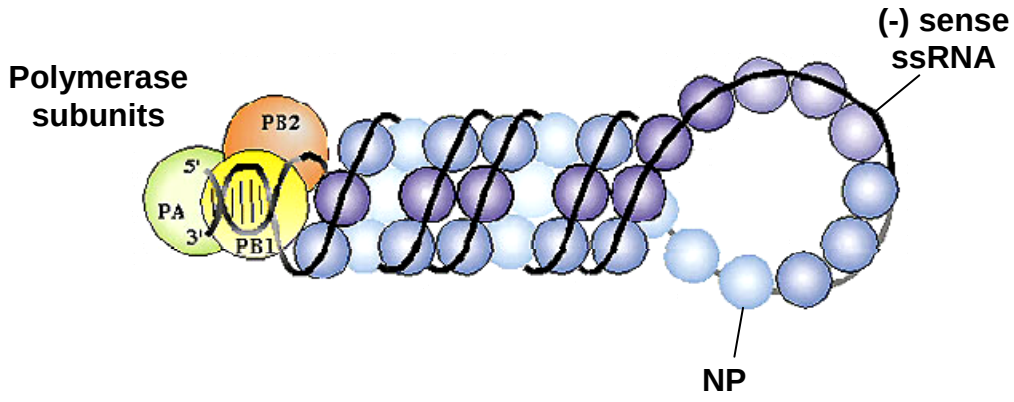


Figure 1.2 Model of the influenza virus RNP structure. Blue spheres represent NP monomers with associated vRNA molecule (black line). The single-stranded vRNA is coiled into a hairpin structure with a short region of duplex (formed between the 5′ and the 3′ ends) which forms the binding site for the heterotrimeric RNA-dependent RNA polymerase. Adapted from Portela and Digard 2002. http://wwwmicro.msb.le.ac.uk/3035/Orthomyxoviruses.html

1.1.3 Replication of influenza A viruses

Influenza viruses enter cells by binding to sialic acid residues present on cell surface glycoproteins or glycolipids via the receptor-binding site in the distal tip of the HA molecules, and are engulfed in endosomes, a process called "receptor-mediated endocytosis". Following internalization and fusion with endosomes, RNP complexes are dissociated from M1 proteins and liberated to the cytoplasm mediated by acidic pH changes in the endosome. RNPs then enter the nucleus through the nuclear pore (Martin and Helenius 1991). The transcription and replication for influenza virus genome occur in the nucleus of infected cells. The viral RNAs (vRNAs) are transcribed into viral mRNAs and replicated by the viral polymerase complex. The genome of influenza virus is negative-stranded RNA. vRNAs are templates for the synthesis of both mRNAs and cRNAs. cRNA is the exact complimentary copy of vRNA and acts as a template for further synthesis of vRNA. All eight vRNA segments contain 12 and 13 conserved nucleotides at their 3' and 5' ends, respectively. In addition, each RNA segment also contains 2 to 3 segment specific nucleotides near each end. These RNA sequences are partially complementary and can form a panhandle structure. Viral mRNA is a capped and polyadenylated transcript. It is an incomplete copy of the vRNA, lacking approximately 17 bases of the complementary viral 3' sequence. Unlike vRNA and mRNAs, cRNAs are not transported to the cytoplasm during viral infection. At the end of the infection cycle, the newly assembled RNPs are exported into the cytoplasm and incorporated into progeny virions, which are released from the cell surface through budding (Fig 1.3)(Lamb and Krug 2001).

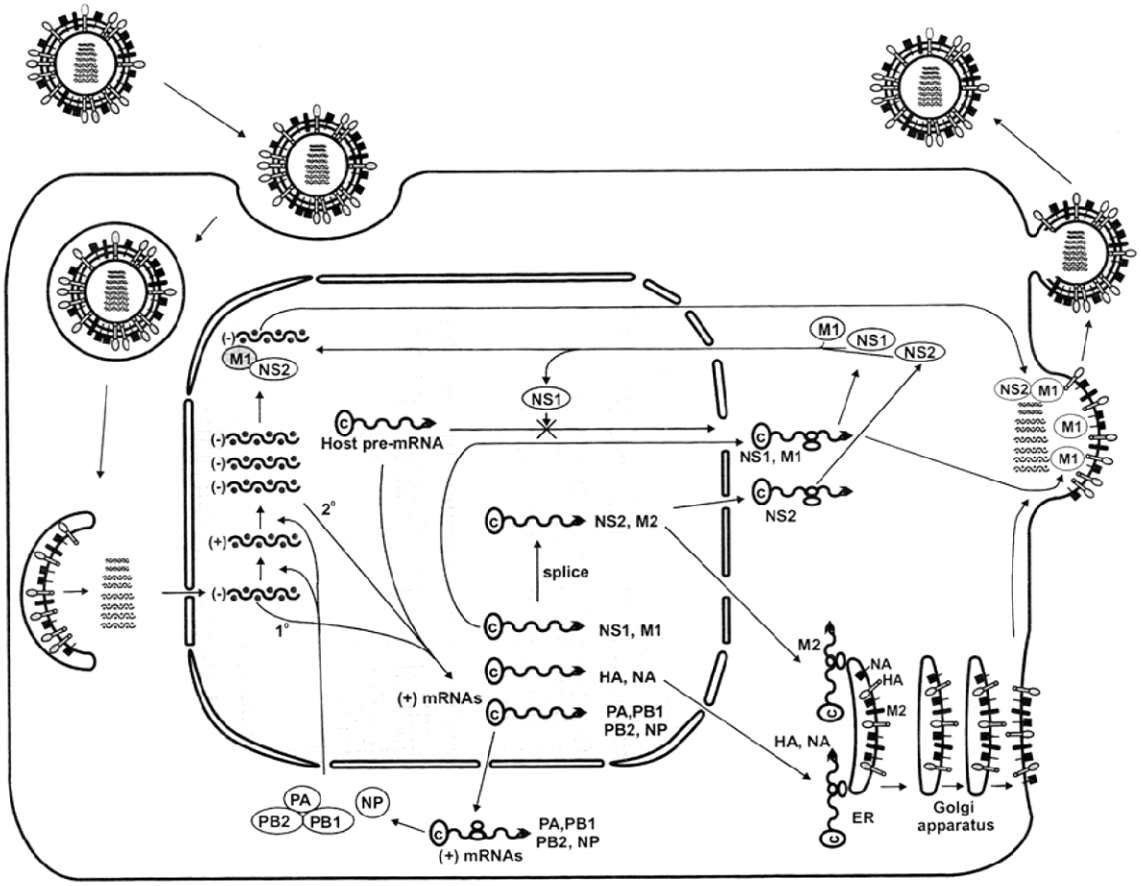


Figure 1.3 Schematic diagram of the life cycle of influenza A virus. Influenza A virus binds to the cell surface and enters the cell by receptor-mediated endocytosis. Following internalization and fusion with the endosome, viral RNPs are released in the cytoplasm. Viral RNPs enter the nucleus through the nuclear pore. In the nucleus of infected cells, the viral RNAs are transcribed into mRNAs and replicated by viral polymerase complexes. The newly assembled viral RNPs are exported into the cytoplasm and incorporated into mature virions which are released from the cell surface by budding. Adapted from Lamb and Krug 2001.

The syntheses of influenza viral mRNAs are initiated by the unique "cap-snatching" mechanism (Figure 1.4). The PB2 subunit of viral polymerase binds to the 5' end of host cell mRNA and cleaves capped (m7GpppNm-containing) cellular RNA 10-13 nucleotides downstream from the 5'end via its cap-dependent endonuclease activity. After cleavage, the short-capped RNA fragment subsequently serves as a primer for initiation of viral mRNA synthesis by the viral polymerase (Plotch et al. 1981). The cap structure (m7GpppNm) from mammalian cellular mRNA is the preferred primer for viral transcription. Although the viral mRNAs also contain the capped structure, they seem to be protected from the endonucleolytic cleavage. These observations suggested that the viral polymerase complex selectively uses host mRNA for viral mRNA synthesis (Shih and Krug 1996). The viral polymerase complex elongates viral mRNA until reaching a stretch of 5 to 7 uridine(U) residues located 15-22 nucleotides before the 5' end of the'vRNA template. After reiteratively copying the U-track at the 5 'end of vRNA template, the viral polymerase generates the 3' polyadenylated tail for the viral mRNAs in a host-independent process (Plotch et al. 1979; Poon 1999).

The viral polymerase is also responsible for the replication of vRNAs in the nucleus (Krug et al. 1989). The replication of vRNAs occurs in two steps: (1) the synthesis of full-length complementary RNAs (cRNAs) by using vRNAs as templates; and (2) the synthesis of full-length progeny vRNAs by copying cRNAs. Unlike viral mRNA synthesis, the synthesis of cRNAs and vRNAs is initiated *de novo* without a primer (unprimed initiation), and is not terminated at the poly (A) site (antitermination). It has not been determined what triggers the switch of influenza viral polymerase activity from capped RNA-primed transcription to unprimed viral RNA replication. However, the free NP proteins not associated with nucleocapsids are required for the specific encapsidation of vRNAs and cRNAs but not mRNAs. Furthermore, the function

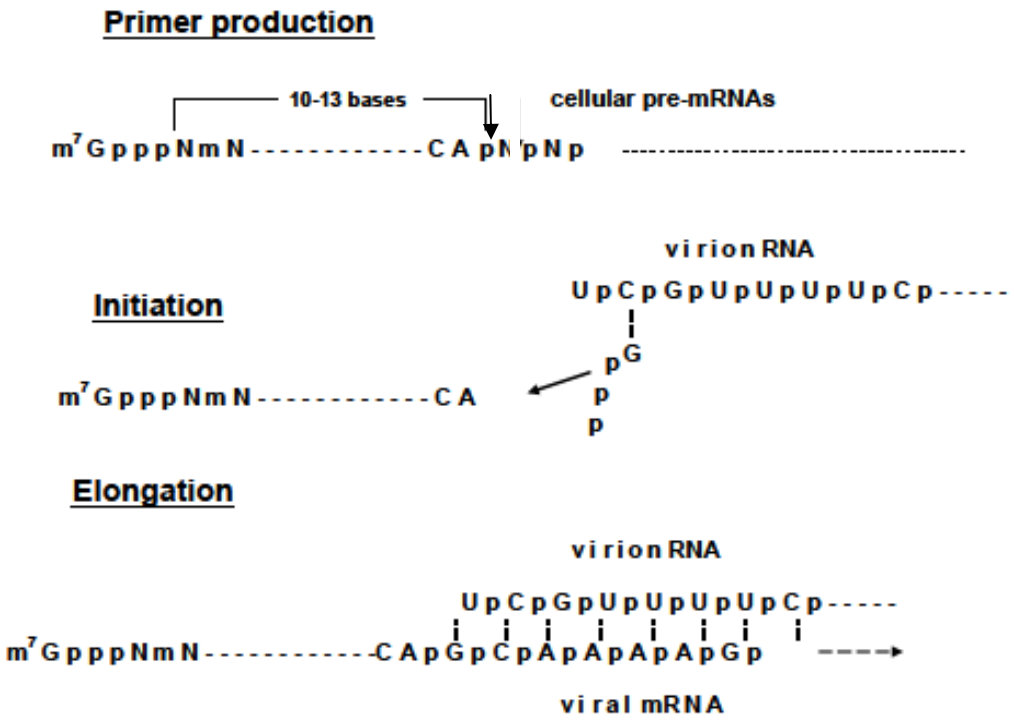


Figure 1.4 **"Cap-snatching" by the influenza viral polymerase.** See text for details. Adapted from Chen and Krug 2000.

of free NP protein is required for: 1) antitermination at poly (U) stretch as part of the switch from mRNA to full-length cRNA synthesis (Beaton and Krug 1986), and 2) synthesis of vRNAs from cRNA templates (Shapiro and Krug 1988).

Viral gene expression in influenza A virus infected cells is regulated over the time course of infection. In general, the amount of viral proteins synthesized in infected cells is largely dependent on the amount of the corresponding mRNA in infected cells. Primary transcription occurs immediately after infection. During this stage, all eight viral mRNAs are synthesized in equimolar amounts, and the synthesis of vRNAs, viral mRNAs, and viral proteins are coupled. Second transcription stage occurs subsequently and can be further divided into early and late phases (Shapiro et al. 1987; Shapiro and Krug 1988). In the early phase, the synthesis rates of both viral mRNA and vRNA increase simultaneously; the late phase is defined by the decrease in the rate of viral mRNAs synthesis while the synthesis of all vRNAs still remain at or near the maximum during this entire period (Shapiro and Krug 1988). Also, in the early phase of the secondary transcription, NS1 and NP vRNA are preferentially synthesized. As a consequence, NS1 and NP are the predominant viral proteins in infected cells at this stage. The reason for the preferential early expression of the NS1 and NP proteins might be that NP is required for the replication and transcription of viral RNA and NS1 is required for the regulation of cellular gene expression. During the late phase, vRNAs are synthesized in equivalent amounts, as required for progeny virus genome. At this stage, the NS1 protein is synthesized in a reduced level, but HA, NA, and M1 mRNAs are preferentially expressed. Capped and polyadenylated viral mRNAs are transported from the nucleus to the cytoplasm for protein synthesis. The membrane-bound proteins, such as HA, NA, and M2, are post-translationally glycosylated in the trans-Golgi network (Figure 1.3), and are transported to the cell surface for incorporation into the cell membrane.

1.1.4 NS1 protein of influenza A virus (NS1A protein)

The non-structural NS1 protein of influenza A virus (NS1A) is a multifunctional protein that participates in protein-RNA and protein-protein interactions (Krug et al. 2003). The NS1A protein, and also the NS2A protein, is encoded by the smallest genomic RNA segment 8, which contains ≈ 890 nucleotides (Lamb and Choppin 1979). The NS1A protein is encoded by the collinear mRNA transcript, whereas a spliced mRNA (NS2A mRNA) encodes the NS2A protein. The NS1A and NS2A proteins share the same 10 amino acids at their N-terminus (Fig 1.5). Subsequent amino acids differ because the NS2A reading frame after the 3' splice site is +1 relative to that of NS1A. Splicing is inefficient, resulting in a 10:1 ratio of unspliced NS1A mRNA: NS2 spliced mRNA, and the proteins are synthesized in the same relative amounts The NS1A protein, approximately 26kDa, contains 202 to 237 amino acids among different influenza A virus strains. It is designated as a "nonstructural" protein because it is synthesized in large amounts in infected cell, but not incorporated into virions (Lazarowitz et al. 1971).

The NS1A protein is composed of two functional domains (Qian et al.1994): the N-terminal first 73 amino acids form the RNA-binding domain (RBD) of a symmetric homodimer with a unique six-helical chain fold (Liu et al. 1997; Chien et al. 1997; Yin et al. 2007); and the remaining two-thirds carboxyl terminus forms the effector domain . As determined by both nuclear magnetic resonance (NMR) and X-ray crystallography, NS1A RBD forms a symmetrical homodimer with a unique six-helical chain fold (Figure 1.6A), which differs from that of the predominant class of dsRNA-binding domains that are found in a large number of cellular proteins (Qian et al. 1995; Chien et al. 1997; Liu et al. 1997; Wang et al. 1999; Yin et al. 2007).

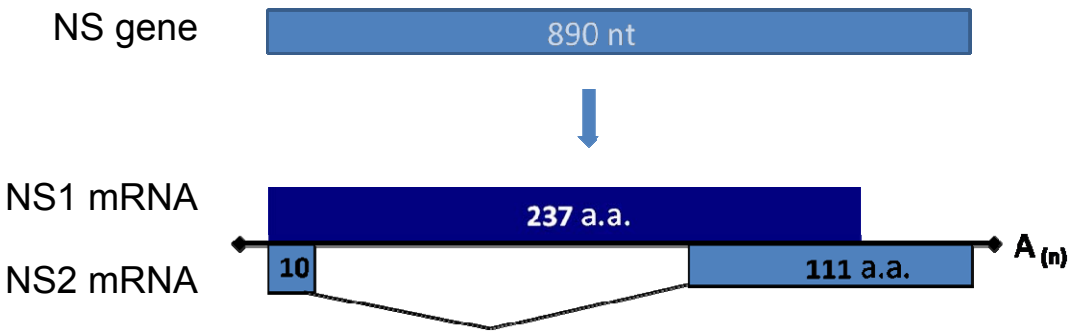
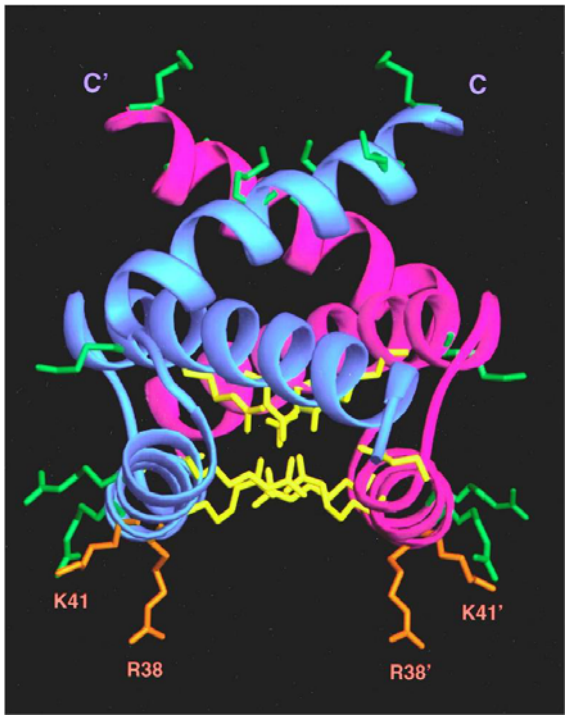


Figure 1.5 **Alternative splicing of the NS2 mRNA.** The NS1A protein is encoded by the collinear mRNA transcript, whereas a spliced mRNA (NS2 mRNA) encodes the NS2A protein. The NS1A and NS2A proteins share the same 10 amino acids at their N-terminus Subsequent amino acids differ because the NS2A reading frame after the 3' splice site is +1 relative to that of NS1A.

(A)



(B)

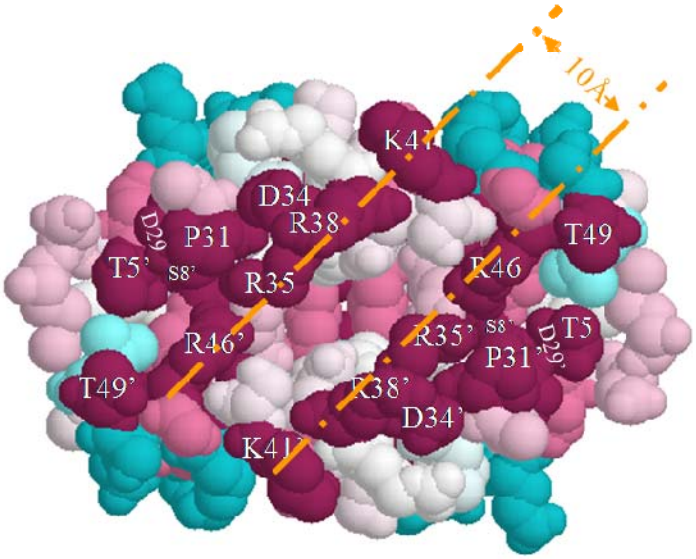


Figure 1.6 **Models of the RNA-binding domain of NS1A protein.** (A) The three-dimensional structure of RNA-binding domain of the NS1A protein. Amino acids R38, R38', K41, and K41' that are crucial for RNA binding are shown as indicated. (B) Model of the RNA binding groove for the RBD dimer.

According to the gel filtration and sedimentation equilibrium measurements, the dimer of NS1A RBD binds to the 16 base-pair synthetic dsRNA duplex with an apparent dissociation constant (Kd) of approximately 1 μM. By comparison, the predominant class of cellular dsRNA-binding proteins containing one or more dsRBMs has approximately 5,000-fold higher affinity for dsRNA (Tian and Mathews 2001). Although the NS1A RBD binds dsRNA with low affinity, it already accounts for all of the dsRNA binding activities of the full-length protein (Qian et al. 1995).

Each polypeptide chain of the NS1A RNA-binding domain consists of three α-helices: Helix1, residues Asn4-Asp24; Helix 2, Pro31-Leu50; and Helix 3, Ile54-Lys 70 (Wang et al. 1999; Chien et al. 2004). The symmetrical homodimer structure is required for dsRNA binding, and the antiparallel helices 2 and 2' play a central role in binding the dsRNA target. The arginine side chain at position 38 (R38) in the second α-helix is the only amino-acid side chain that is absolutely required for dsRNA binding, and the lysine side chain at position 41 (K41) also contribute to the binding affinity (Wang et al. 1999). Furthermore, NS1A RBD binds to canonical A-form dsRNA, but does not bind to dsDNA or dsRNA-DNA hybrids, which feature B-type or A/B-type intermediate conformations, respectively (Chien et al. 2004). The current hypothetical model is that NS1A RBD sits astride the minor groove of A-form RNA with a few amino acids in the helix 2-helix 2' face forming an electrostatically stabilized interaction with the phosphodiester backbone (Figure 1.6(B)). This mode of dsRNA binding differs from that observed for any other dsRNA-binding protein. Although NS1A RBD also binds *in vitro* to a specific stem-bulge of U6 snRNA (Qiu and Krug 1994; Qiu et al. 1995), and binds with a lower affinity to a specific stem-bulge in the minor U6 atac snRNA (Wang and Krug 1998), no biological role of this binding has been found.

The primary function of the NS1A RNA-binding domain has been elucidated. Early studies suggested that the NS1A RNA-binding domain acts by sequestering viral dsRNA away from, and inhibit the activation of, the endogenous cellular PKR kinase (Nemeroff et al. 1995; Lu et al. 1995). Research conducted in our laboratory using a recombinant influenza A/Udorn/72 virus expressing a NS1A protein lacking dsRNA-binding activity, i.e., a virus encoding a NS1A protein with a R38A amino acid substitution, clarified two controversial aspects of the functions of this domain. First, NS1A protein inhibits the activation of PKR, not by sequestering dsRNA, but by direct binding via its effector domain to the N-terminal region of PKR (Li et al. 2006; Min et al. 2007). Second, the dsRNA-binding activity of NS1A protein does not have a role in inhibiting the influenza A virus-induced synthesis of IFN-β mRNA. Rather, the primary function of the NS1A dsRNA-binding activity is to protect influenza A virus against the antiviral state induced by IFN-beta by inhibiting the IFN-α/β-induced 2'-5'-oligo (A) synthetase (OAS)/RNase L pathway (Min and Krug 2006).

The C-terminus of the NS1A protein forms the effector domain, extending from the end of the RNA-binding domain to the carboxyl terminal amino acid (Qian et al. 1994; Chen and Krug 2000). NS1A effector domain forms dimers (Bornholdt and Prasad 2006; Hale et al. 2008) (Figure 1.7). Each monomer consists of seven β-strands and three α-helices. Six of the β-strands form an antiparallel β-sheet, but not the last one. The β-sheet surrounds the long α-helix, which is held in the central through hydrophobic interactions between the β-sheet and the α-helix. This structure is described as a "α-helix β-crescent fold". Different models have been proposed for the formation of the effector domain dimer. In Prasad's model (Figure 1.7(B)), the interface of the effector domain dimer, PR8/NS1Δ72, is formed by the first N-terminal β-strand of each monomer (strand-strand dimer). These β-strands run antiparallel to each other along the dimeric interface.

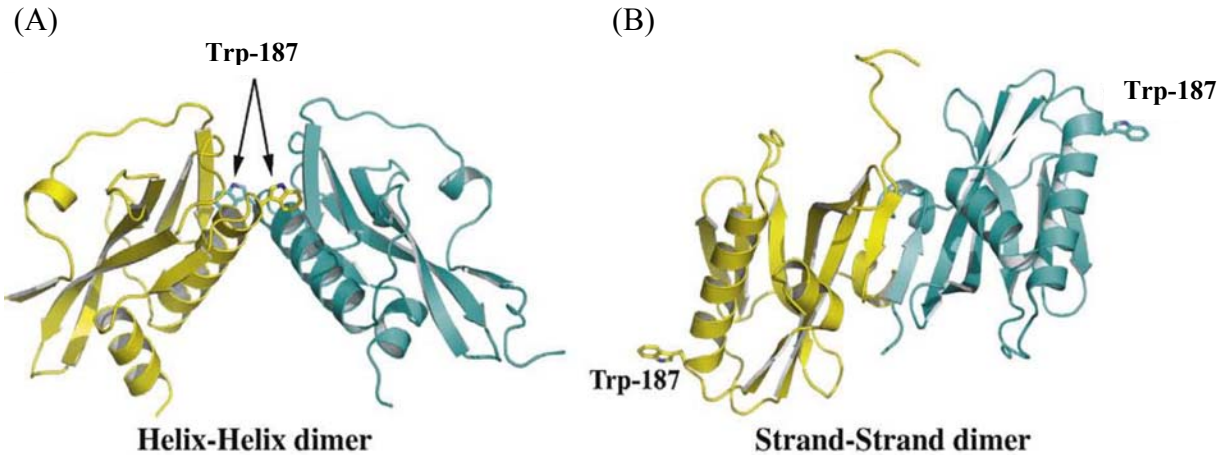


Figure 1.7 Models of the dimeric structure of NS1A effector domain. (A) Helix-to-helix dimer. Hale's model. The interface of the effector domain dimer , NS1A, Alb/ NS1Δ72, is between the two long α-helices of the monomers, and Trp-187 is essential for the dimerization. (B) Strand-to-strand dimer. Prasad's model. The interface of the effector domain dimer, PR8/NS1Δ72, is formed by the first N-terminal β-strand of each monomer (strand-strand dimer). These β-strands run antiparallel to each other along the dimeric interface.

In contrast, in Hale's model (Figure 1.7(A)) the effector domain dimer of an avian influenza virus NS1A, Alb/ NS1Δ72, shows the interaction between the two long α-helices of the monomers, and Trp-187 is essential for the dimerization. There are still possibilities that there are functional roles for both dimeric states, which occur in either a strain-specific or ligand-dependent manner. Thus, NS1A proteins may exhibit variation in their dimeric assemblies, a property that could contribute to their apparent multifunctional nature. It should also be noted that dimerization of the isolated NS1A protein effector domain may differ from that observed in the full-length NS1A protein, whose overall conformation is influenced by both the RNA binding and effector domains (Nemeroff et al. 1995).

Because of the intrinsic protein disorder of the C-terminal "tail" residues downstream of Ala-202 or Asn-207, this "tail" region was not resolved in either Hale's or Prasad's models. The structure and functions of this region of NS1A are intriguing. Previously NS1A proteins have been described that they either lack up to ~20 residues of this "tail", or have a 7 amino-acid extension (Suarez and Perdue 1998). In addition, several functions have been assigned to this region in avian NS1A proteins but not in human NS1A proteins. For example, avian NS1A proteins contain a consensus Src homology 3 (SH3) domain-binding motif at residues 212–215, which can interact with human Crk/CrkL proteins to enhance cellular PI3K activation (Heikkinen et al. 2008). In addition, as described in the following chapters, the Thr at 215 in human NS1A proteins is phosphorylated during infection, and this phosphorylation is required for optimal virus replication. Furthermore, the four C-terminal residues of most avian NS1A proteins form a putative PDZ domain ligand (Obenauer et al. 2006), and these residues of NS1A modulate virus pathogenicity in mice (Jackson et al. 2008).

The effector domains has binding sites for several cellular proteins that mediate various functions (Fig 1.8): (i) the inhibition of the cellular pre-mRNA 3'- end processing machinery by binding to the 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II (PABII) (Nemeroff et al. 1998; Chen et al. 1999; Li et al. 2001; Noah et al. 2003; Twu et al. 2006;); (ii) inhibition of the activation of dsRNA-activated protein kinase (PKR) by direct binding of PKR(Li et al. 2006; Min et al. 2007); and (iii) the activation of phosphatidylinositol-3-kinase (PI3K) signaling by binding to p85β subunit of PI3K (Hale et al. 2006; Shin et al. 2007a; Ehrhardt et al. 2007).

A major function of the NS1A protein probably is the inhibition of the antiviral effects of interferon (IFN). The NS1A protein inhibits the production of mature cellular mRNAs, including IFN-β mRNA, through its effector domain, by binding to two cellular proteins that are essential for the 3'end processing of cellular pre-mRNAs: the 30kD subunit of CPSF (Nemeroff et al. 1998) and PABII (Chen et al. 1999). The interactions were discovered by yeast two-hybrid screening, and the binding sites of these two proteins on NS1A, determined by GST-pulldown experiments, do not overlap each other: CPSF 30kD subunit binds to the region with amino acid 186 as the center; PABII binds to the region from amino acid 223 to 237 (Li et al. 2001). In the 3' end pre-mRNA processing machinery, the CPSF factor recognizes and binds to the AAUAAA poly (A) signal, which is required for both cleavage and polyadenylation of cellular pre-mRNAs (Colgan and Manley 1997), and PABII stimulates the synthesis of poly (A) tails for cellular mRNA catalyzed by poly (A) polymerase (PAP) (Chen et al. 1999). Binding of NS1A protein to the CPSF 30kD subunit and PABII prevents their functions and results

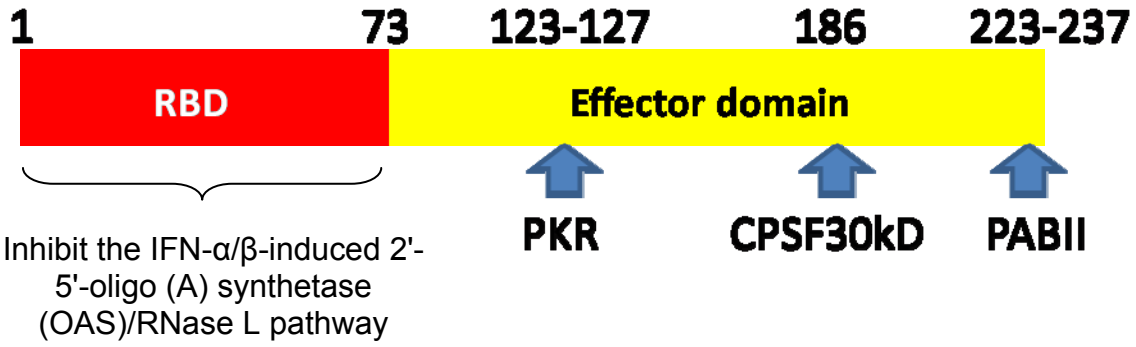


Figure 1.8 Schematic diagram of NS1A protein structure. Binding sites of the cellular proteins on the NS1A effector domain are shown as indicated.

in the accumulation of cellular pre-mRNA containing short poly (A) tails of approximately 12 nulceotides in the nucleus of infected cells (Nemeroff et al. 1998), where they would be more accessible to the viral cap-dependent endonuclease for the production of the capped RNA primers needed for viral mRNA synthesis (Krug 1989). (Figure1.9). The poly(A) tails of viral mRNAs are added by the influenza viral polymerase, so the inhibition of the cellular 3'end processing machinery by NS1A protein does not affect the production of mature viral mRNA (Poon et al. 1999). It has also been shown that, *in vitro*, NS1A protein inhibits the splicing of cellular pre-mRNAs by binding to a specific stem-bulge of U6 snRNA which is an important factor in pre-mRNA splicing (Qiu et al. 1995). However, whether such inhibition also occurs in infected cells has not been established, since all of the sequestered cellular pre-mRNAs in the nucleus eventually would be degraded rapidly (Katze and Krug 1984). The inhibition of 3'end processing is crucial, since influenza A virus can activate the RIG-I RNA helicase to trigger the activation of IRF-3 and NF-κB and hence the synthesis of IFN-β pre-mRNA (Siren et al. 2006).

The NS1A protein also inhibits the activation of Ser/Thr protein kinase, PKR, during virus infection. PKR is activated by binding to either dsRNA or the cellular PACT protein. Activated PKR phosphorylates the translation initiation factor eIF2α, thereby inhibiting viral and cellular protein synthesis and virus replication (Sadler and Williams 2008; Sarkar and Sen 2004). As described above, instead of competing for the dsRNA binding, the NS1A protein directly binds to PKR and inhibits its activation mediated by either PACT or dsRNA. The interaction region has been determined by GST pull-down experiments: amino acids 123-127 of the NS1A protein interact with the N-terminal 170-230 amino acid region of PKR. (Tan and Katze 1998; Li et al. 2006; Min et al. 2007). By using a recombinant influenza A/udorn/72 virus encoding a NS1A protein in which

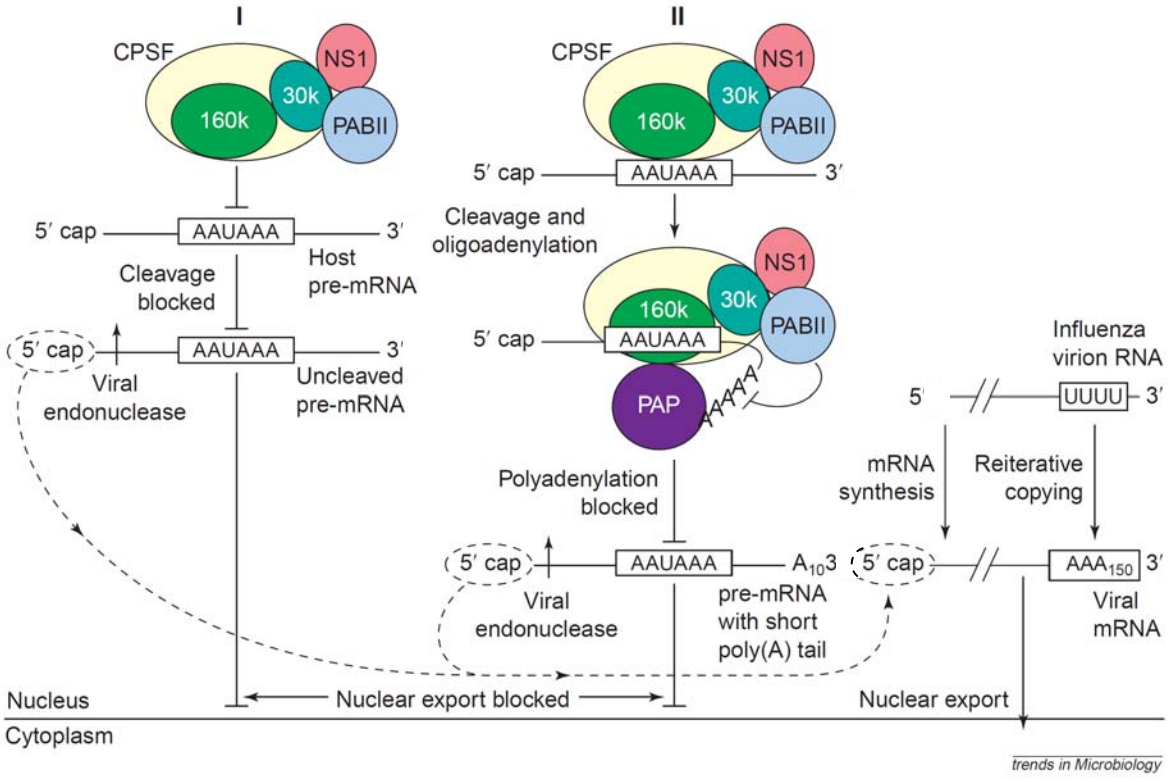


Figure1.9 **Mechanisms by which NS1A inhibits the cellular 3'end processing system in influenza-virus-infected cells.** Pathway I: The binding of the NS1 protein to the 30kDa subunit of CPSF blocks the CPSF binding to the AAUAAA sequence of some cellular pre-mRNA molecules, thereby blocking 3' cleavage pre-mRNAs. The uncleaved pre-mRNAs remain in the nucleus. Pathway II: CPSF binds to the AAUAAA sequence of other cellular pre-mRNA molecules, despite the binding of the NS1A protein to the 30kDa CPSF subunit. A short poly(A) sequence is then added to these cleaved pre-mRNAs by PAP in a CPSF-dependent reaction. Subsequent elongation of the short poly(A) sequence is blocked by the binding of the NS1A protein to the PABII protein, resulting in the nuclear accumulation of cleaved pre-mRNAs containing short poly(A) tail. The newly synthesized pre-mRNAs sequestered in the nucleus are cleaved by the viral cap-dependent endonuclease to produce the primers required for viral mRNAs synthesis. The 3' poly(A) sequence on viral mRNAs is produced by the viral polymerases and the poly(A)-containing viral mRNAs are exported from the nucleus. Adapted from Chen and Krug 2000.

amino acids 123 and 124 were changed to alanines (123/124 AA), PKR is not activated *in vivo*. The NS1A protein has been reported to interact with eIF4GI, the large subunit of the cap-binding complex eIF4F, and poly(A) binding protein I (PABP1)(Aragon et al. 2000; Burgui et al. 2003), and to recruit them specifically to the 5' UTR of viral mRNAs, resulting in the preferential translation of influenza viral mRNAs. However, this observation has not been verified by others.

It has also been suggested that the NS1A protein interacts with the viral polymerase. It was reported that the three subunits of the influenza virus polymerase and NP can be co-immunoprecipitated with NS1A by anti-NS1A serum (Marion et al. 1997), This co-immunoprecipitation was insensitive to RNase treatment, indicating that the NS1A-polymerase interaction was not mediated by RNA, but resulted from a direct protein-protein interaction. One of my goals was to devise a better strategy to establish whether a NS1A-polymerase interaction occurs in infected cells (Chapter 3).

The effector domain of NS1A proteins contains SH3-binding motifs, which mediate binding to cellular proteins. The NS1A class I SH3 binding motif (amino acids 164-167), which is found in human and avian NS1A proteins, binds the p85β subunit of phosphoinositide 3-kinase (PI3K), resulting in the the PI3K-dependent phosphorylation of Akt (protein kinase B) (Shin et al. 2007; Hale et al. 2008; Li et al. 2008). The class II SH3 motif (amino acids 211-216) of NS1A protein, which is very common among avian strains but rarely found in human strains, binds to the adapter protein Crk/CrkL (Heikkinen et al. 2008). .This binding apparently enhances PI3K/Akt activation in avian influenza A virus infected cells. Activation of the PI3K/Akt pathway induces phosphorylation of caspase-9 and thereby inhibits viral-induced apoptosis, resulting in efficient virus replication (Ehrhardt et al. 2007; Shin et al. 2007). However, it is also know that NS1A protein induces apoptosis in various cells, and that caspase-3 activation

is important for efficient virus replication (Wurzer et al. 2003) as apoptosis promotes caspase-dependent migration of virus RNP complexes to the cytoplasm for efficient packaging into progeny virions. It is not surprising that influenza viruses have evolved to take advantage of existing complementary host cell responses to support their propagation. The mechanism which causes NS1A to switch from anti-apoptotic effects at early times of infection to pro-apoptotic effects later in infection remains to be elucidated.

In addition to the important interacting partner described above, by employing yeast 2-hybrid system, three other cellular proteins were also shown to interact with NS1A: NS1-I, a human homolog of the porcine 17β−estradiol dehydrogenase precursor protein (Wolff et al. 1996); NS1-BP, a human 70-kDa protein (Wolff et al. 1998), and hStaufen, a human homologue of *Drosophila melanogaster* Staufen protein (Falcon et al. 1999). These interactions were confirmed by either GST-pull down assay, co-localization in cells, or co-immunoprecipitation, but they have not been shown to play a functional role in influenza A virus-infected cells.

Although the NS1A protein is a relatively small protein with a molecular weight of approximately 26kDa, it has been reported to bind to a number of very different host factors and function through various pathways to support viral propagation. In addition to basic protein-protein interaction, there could be other biological activities involved in regulating the functions of NS1A, for example, phosphorylation, as described below.

1.1.5 NS1A phosphorylation

Multifunctional proteins are often regulated by post-translational modifications (PTMs). Advances in mass spectrometry analysis have allowed the systematic screening of various PTMs on proteins (Witze et al. 2007). About one-third of eukaryotic proteins

are phosphorylated, and protein phosphorylation has been implicated to regulate a wide variety of cellular processes. Numerous viral proteins have been reported to be phosphorylated. Phosphorylation of viral proteins has been shown to regulate different aspects of the virus life cycle, e.g., activation of transcription, change of cellular sub-localization, change of protein-protein interactions, or change of protein stability (Lenard 1999; Jakubiec and Jupin 2007).

In addition to its RNA- and protein-binding activities, the NS1A protein is phosphorylated during infection (Privalsky and Penhoet 1978; Privalsky and Penhoet 1981; Petri et al. 1982; Skorko et al. 1991). Other influenza A virus phosphoproteins include NP, NS2, M1, PA, and M2 (Privalsky and Penhoet 1977; Gregoriades et al. 1984; Skorko et al. 1991; Richardson and Akkina 1991; Holsinger et al. 1995; Arrese and Portela 1996; Sanz-Ezquerro et al. 1998; Thomas et al. 1998; Reinhardt and Wolff 2000; Huarte et al. 2003; Wu and Pekosz 2007). The phosphorylation state of NP and M1 is known to control their intracellular distribution (Neumann et al. 1997; Whittaker et al. 1995). The functional association between M1 and NP is also suggested to be regulated by phosphorylation. Meanwhile, the role of NS1A phosphorylation during virus infection is not known.

NS1A phosphorylation was first described 30 years ago (Privalsky and Penhoet 1978). It was demonstrated by pulse-labeling infected cells with ^{32}P-orthophosphate, and two-dimensional gel electrophoresis of labeled proteins. Two phosphorylated species of the NS1A protein were found. One species was phosphorylated at one site, and the other one contained two phosphorylation events. Thin-layer electrophoresis analysis of phosphoaminoacids established that NS1A was phosphorylated at two threonine residues (Privalsky and Penhoet 1981). NS1A phosphorylation is strain-specific and was not detected in avian NS1A proteins (Petri et al. 1982). NS1A phosphorylation is resistant to

bacterial alkaline phosphatase treatment. It is believed that phosphate in the intact NS1A protein is in the form of terminal phosphomonoesters which are protected from enzymatic hydrolysis. Neither the exact residues that are phosphorylated nor the role of this posttranslational modification in influenza virus replication has yet been determined.

1.1.6 Phosphorylation of other viral proteins

Phosphorylation is a major post-translational protein modification event and plays an important role in regulating multiple cellular processes. Phosphorylation could affect proteins by modulating their enzymatic activity, stability, cellular localization or interactions with other proteins.

It has been reported that several viral proteins are phosphoproteins. The P protein of vesicular stomatitis virus (VSV) and the NS5A nonstructural protein of hepatitis C virus (HCV) are two widely studied examples. The primary phosphorylation of P protein occurs between residues 35 and 78. This phosphorylation of P protein is essential for the activation of transcription. Caesin kinase 2 (CK II) is the major cell kinase for VSV P protein phosphorylation and is responsible for the constitutive primary phosphorylation. The primary phosphorylation of P protein by CK II is necessary and sufficient for transcriptional activation by promoting the formation of a P protein homotrimer, which enables the P protein to associate with L protein and bind it to the nucleocapsid-associated RNA template. The sites of secondary phosphorylation on P protein locate within residues 212-244 near the C-terminus of the P protein. The kinase that is involved and the functions of secondary phosphorylation are not clear. It has been proposed that secondary phosphorylation of VSV P protein might shut off viral replication prior to assembly of progeny viruses (Lenard 1999). NS5A protein of hepatitis C virus is a

multifunctional phosphorylated nonstructural protein involved in regulating viral replication and virus assembly. NS5A is phosphorylated on multiple serine and threonine residues. It exists as two phosphorylated forms, basally phosphorylated (p56) and hyperphosphorylated (p58) proteins. The basally phosphorylated form is a pre-requisition for establishing the hyperphosphorylated state. NS5A phosphorylation is an important regulatory mechanism for the viral RNA replication. Reduction of hyperphosphorylated form of NS5A correlates with enhanced replication of an HCV replication in tissue culture. It has been suggested that this effect is due to the disruption of interaction with the docking protein hVAP-A (human vesicle-associated membrane protein-associated protein A), which impairs the assembly of viral replication complexes. A critical ratio between the p56 and p58 phosphoforms of NS5A is required for productive HCV RNA replication (Huang et al. 2007). Furthermore, a recent report indicates that serine 457 of NS5A is a CK II phosphorylation site and phosphorylation at this site is required for infectious virus production (Tellinghuisen et al. 2008).

1.2 CELLULAR ANTIVIRAL RESPONSE

Host cells launch immune responses to counteract various invasions of pathogens. The immune responses include an early non-specific innate mechanism and a specific adaptive mechanism. Innate immune response is the first line of defense against viral infection.

1.2.1 IFN-independent early antiviral response

During the initial viral infection, a number of antiviral genes are induced by activating transcription factors controlling their expressions. This early antiviral response occurs prior to the production of interferons and does not require viral protein synthesis

(Boyle et al. 1999; Chin and Cresswell 2001; Navarro et al. 1998; Preston et al. 2001; Wathelet et al. 1998; Weaver et al. 1998; Yoneyama et al.1998; Parekh and Maniatis 1999; Hiscott et al. 1999; Au et al. 1995; Kim et al. 2002; Noah et al. 2003). Upon virus infection, two transcriptional factors, interferon regulatory factor-3 (IRF-3) and IRF-7, are activated by phosphorylation and translocated into the nucleus. IRF-3 and IRF-7 combined with the transcriptional coactivators p300 and CREB-binding protein form a transcription complex which is called virus-activated factor (VAF), and this complex binds to the IFN-stimulated response elements (ISREs) in the promoters of particular cellular antiviral genes, thereby inducing their transcription. The antiviral proteins produced through this early IFN-independent immune response provide initial protection of the infected cells against viral replication. (Kim et al. 2002; Simmen et al. 2001).

1.2.2 Antiviral response mediated by interferons

Interferon (IFN) is a key component of the host defense response (reviewed in (Randall and Goodbourn 2008; Sadler and Williams 2008). The production and secretion of IFN occur in response to various external stimuli specifically including virus infection. IFNs bind to the receptors on cell surface and activate cellular signaling cascades which lead to the limitation of further spread of the disease. There are three types of interferons, designated as type I, II, and III, according to their amino acid sequences and the cell surface receptors that they bind. Type I IFNs in human contain 13 IFNα subtypes, IFNβ, IFNδ, IFNε, IFNκ, IFNo, and IFNτ. Type I IFN binds to the ubiquitously expressed heterodimeric receptor, IFNAR (IFNα receptor), which is consist of IFNAR1 and IFNAR2. The type II IFN only has single IFNγ gene, and is secreted by T cells and NK cells. The dimer of IFNγ proteins binds to a tetramer consisting of 2 IFNGR2 (IFNγ

receptor 2) and 2 IFNGR1 chains. Type III IFNs have three IFNλ subtypes (IFNλ1, IFNλ2, IFNλ3, also referred to as IL-29, IL-28A, and IL-28B, respectively) that signal through IFNLR1 (IFNλ receptor 1; also known as IL-28Rα) and IL-10R2 (interleukin-10 receptor 2; also known as IL-10R). Type III IFNs are also induced directly in response to viral infection and is thought to be the prototype of type I IFNs.

IFN α and β of type I IFNs play an essential role in innate immunity. IFN α/β are rapidly induced in direct response to many viral infections. Cells secreting IFN-α/β have pattern-recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) such as viral dsRNA. Once activated, these PRRs induce the signaling cascades leading to the transcription of IFN-α/β. The simplest form for IFN-β induction requires IFN regulatory factor-3 (IRF-3) and nuclear factor kappa B (NF-κB). Upon stimulation, the cytoplasmic IRF-3 is phosphorylated, dimerized, and translocated into the nucleus. The inhibitor molecule, inhibitor of NF-κB (IκB) that retains NFκB in the cytoplasm, is also phosphorylated. The subsequent ubiquitination and degradation of IκB frees NFκB and results in the translocation of NFκB into the nucleus. IFN-β induction also requires the binding of a c-jun/ATF-2 heterodimer. The IRF-3, NFκB, and c-jun/ATF-2 complexes assemble and form the enhanceasome on the promoter. The enhanseasome facilitates the recruitment of CREB-binding protein (CBP)/p300 and promotes the assembly of the basal transcriptional machinery and RNA polymerase II.

IFNα/β exerts its effects through binding to the IFN-α/β receptors on neighboring uninfected cells as well as the initial infected cells resulting in the activation of JAK/STAT-dependent and STAT-independent pathways and the subsequent transcriptional up-regulation of several hundred IFN-stimulated genes (ISGs). Prior to activation, tyrosine kinase 2 (Tyk2) and the tyrosine kinase JAK1 associate with IFNAR1 and IFNAR2, respectively. IFN binding to the receptor causes the dimerization of the

receptor and activates Tyk2 and JAK1 which phosphorylate STAT1 and STAT2 subsequently. Phosphorylated STAT1 and STAT2 form a stable heterodimer and translocate into the nucleus where they associate with a monomer of IRF-9 to form the ISGF3 heterotrimer that binds to the IFN-stimulated response element (ISRE), present in the promoters of most IFN-responsive genes, and induces the transcription. Type III IFN signaling is similar to type I IFN. In type II IFN signaling, subunits of IFNγ receptor, IFNGR1 and IFNGR2, are associated with JAK1 and JAK2, respectively. IFNγ binding to the receptor activates JAK1 and JAK2 and results in the formation of STAT1-STAT1 homodimer that translocates into the nucleus and binds to the gamma-activation sequence (GAS) of ISGs to stimulate the transcription (Figure 1.10).Binding of this homodimer to GAS does not require IRF-9. However, IFNγ can activate several ISRE-containing promoters, including CXCL10, by activating the formation of a heterotrimer containing IRF-9 and STAT1-STAT1 homodimer.

Type I IFNs induced more than 300 ISGs (Der et al. 1998). The antiviral state is established by the combination of these ISGs which involves multiple overlapping or related pathways. Many of them are proteins encode pattern recognition receptors (PRRs) or transcription factors that form the positive feedback loop to limit the virus spread by detecting viral molecules and increasing the production of IFNs. Some of these ISGs encode proteins with the potential of direct antiviral activities through cytoskeletal remodeling, apoptosis induction, post-translational modification, or regulation of post-transcriptional events. Several of these ISG proteins with potential antiviral activities used in my study are described in the following sections.

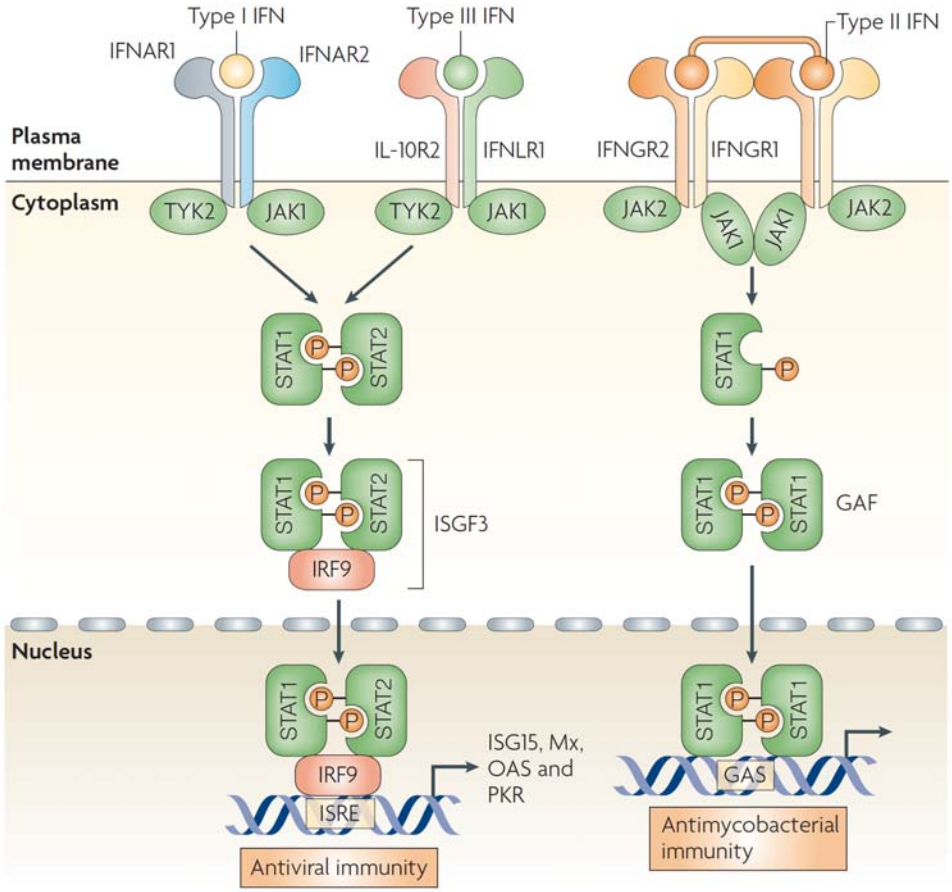


Figure 1.10 Interferons signaling through specific receptors. See text for details. Adapted from Sadler and Williams (2008).

MxA GTPases.

The Mx proteins are members of the superfamily of dynamin-like guanosine triphosphatases (GTPases) and are induced exclusively by type I IFN (Haller et al. 2007). Mx proteins are originally identified as IFN-induced proteins with the ability to inhibit RNA virus replication. Mouse has evolved two distinct types of Mx GTPases to control viruses with different intracellular replication sites. The nuclear murine Mx1 confers resistance to orthomyxoviruses, including influenza A virus and Thogoto viruses, known to replicate in the cell nucleus. The cytoplasmic Mx2 inhibits viruses that replicate in the cytoplasm, such as Rhabdoviruses (vesicular stomatitis virus, VSV) and bunyaviruses (La Crosse virus, LACV) (Haller et al. 2007a; Haller et al. 2007b; Dreiding et al. 1985; Stertz et al. 2007). In human cells, the two Mx proteins, MxA and MxB, both locate in the cytoplasm. Human MxA acts against many viruses regardless their intracellular locations for replication, including members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, and picornaviruses (Kochs and Haller 1999; Haller et al. 2007a). However, human MxB has no known antiviral activity. Mouse Mx1 inhibits nuclear primary transcription of the influenza virus RNA, whereas human MxA inhibits influenza virus replication at a later step because primary viral transcription is unaffected (Pavlovic et al. 1992). When artificially targeted to the nucleus, MxA, like Mx1, inhibits primary transcription by binding to the influenza virus polymerase PB2 and nucleocapsid proteins (Zurcher et al. 1992). In general, following viral infection, MxA binds viral nucleocapsids or other essential viral components to trap and block the transport or viral RNA synthesis, depending on their localizations (Kochs and Haller 1999; Kochs et al. 2002). In addition to Mx proteins, IFNα/β also induces the expression of several other guanine-hydrolysing proteins that involve in host resistance to pathogens, including p47 guanylate-binding proteins (GBPs), the p65 GBPs, the very large inducible

GTPases. Only the Mx proteins have more well-characterized antiviral functions. GBP-1 of large GTPases was shown to inhibit replication of VSV and EMCV, but the mechanism is unknown (Anderson et al. 1999).

P56.

ISG56 is one of the most highly induced ISGs in response to IFN, dsRNA, and many viruses (Der et al. 1998; Guo et al. 2000). The gene product, p56, is not detectable in untreated cells. The induction of ISG56 is transient, and the mRNA and protein turn over quickly (Guo et al. 2000b; Guo et al. 2000a). In humans, p56 is structurally related to p60, p58, and p54 proteins, all of which are induced by viral stresses and contain multiple tetratricopeptides (TPR) motifs which facilitate protein-protein interactions in huge complexes. P56 inhibits translation by interacting with the eIF3e/p48 subunit of the eukaryotic translation initiation factor 3 (eIF3) complex (Guo et al. 2000a) and inhibiting its ability to stabilize the eIF2-GTP-tRNAi^{Met} complex (Hui et al. 2003). Therefore, p56 has the ability to suppress translation of both virus and host proteins.

Viperin.

Viperin (also known as CMV-inducible gene 5, cig5) is an IFN-induced cytoplasmic protein involved in the antiviral response. Expression of viperin in cell-culture systems exhibits potent antiviral activity against HCMV (Chin and Cresswell 2001), HCV (Helbig et al. 2005), HIV (Rivieccio et al., 2006), alphaviruses (Zhang et al., 2007), and influenza A virus (Wang et al. 2007). Viperin binds and inhibits the enzyme farnesyl diphosphate synthase (FPPS), which is involved in the biosynthesis of isoprenoid-derived lipids, and thus inhibits influenza A virus release from the plasma membrane by disrupting the formation of lipid rafts (which are the sites for the budding process of influenza viruses) on the plasma membrane (Wang et al. 2007).

Phospholipid scramblase 1 (PLSCR1).

Phospholipid scramblase 1 (PLSCR1) is a calcium-binding protein which either inserts into the plasma membrane or binds DNA in the nucleus depending on the state of its palmyitoylation. It is highly induced by interferons and various growth factors (Dong et al. 2004). PLSCR1 suppresses viral replications of vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) and inhibits accumulation of primary VSV transcripts. The antiviral effect of PLSCR1 correlated with increased expression of a subset of IFN-stimulated genes (ISGs), including ISG15, p54, p56, and GBPs. It is hypothesized that PLSCR1 is required for amplifying and enhancing the IFN response through increased expression of a select subset of potent antiviral genes.

ISG15

ISG15 is another antiviral protein that is highly induced by IFN. It is discussed in detail in the next section.

1.2.3. ISG15 conjugation

ISG15 (Interferon stimulated gene, 15kD) was originally identified as an interferon stimulated gene (ISG) whose expression is highly induced upon interferon treatment (Korant et al. 1984; Blomstrom et al. 1986; Haas et al. 1987). It is one of the earliest ISGs to be induced following IFN treatment or upon viral infection of human cells. ISG15 is also called ubiquitin cross-reactive protein (UCRP) due to its cross-reactivity with ubiquitin antibodies (Haas et al. 1987). ISG15 is an ubiquitin (Ub)-like protein consisting of two ubiquitin homology domains in tandem and shows ~30% (N-terminal domain) and ~ 36% (C-terminal domain) identical to ubiquitin. ISG15 also functions as a cytokine since it can be released from human lymphocytes, monocytes, and

epithelial cells, and induces the production of IFN-γ (Knight and Cordova 1991; D'Cunha et al. 1996; Recht et al. 1991).

A major similarity between ISG15 and ubiquitin is that they both are covalently conjugated to targeted proteins. The first evidence for the existence of ISG15 protein conjugates was discovered in 1992 (Loeb and Haas 1992), and it was noted that ISG15 conjugated proteins are non-covalently associated with the intermediate filament network (Loeb and Haas 1994; Narasimhan et al. 1996). The precursor of ISG15 contains 165 amino acids and is processed to expose the C-terminal sequence LRLRGG (Potter et al. 1999; Perry et al. 1999). After type I interferon stimulation, ISG15 becomes conjugated to a large numbers of cellular proteins. The C-terminal LRLRGG motif of ISG15 is essential to form covalent conjugates with its targets. The kinetics of the ISG15 conjugation suggest the existence of an interferon inducible system, as free ISG15 is synthesized as early as two hours after IFN treatment and increases to maximal expression by 18 hours. In contrast, the time course of ISG15 conjugation is much slower, with ISG15-conjugated proteins only appearing significantly following 12-18 hours of interferon treatment. In A549 cells, about 53% of the total ISG15 is in conjugated form after IFN treatment (Loeb and Haas 1992).

ISG15 conjugation is similar to ubiquitination in that it involves an enzymatic cascade including E1 activating enzyme, E2 conjugating enzyme, and E3 ligase. However, conjugation of ISG15 to intracellular target proteins was shown to be distinct from that targeted by Ub (Narasimhan et al. 1996). Our laboratory identified the E1 and E2 enzymes in the ISG15 conjugation pathway, and showed that they are induced by IFN–α/β. UbE1L (E1-like ubiquitin-activating enzyme), a single-subunit enzyme capable of activating ISG15 but not Ub, is the E1 for ISG15 conjugation (Yuan and Krug 2001). UbcH8 is the E2 for ISG15 conjugation (Zhao et al. 2004; Kim et al. 2004). The

HECT E3 ligase, Herc5 (HECT domain and RCC1-like domain containing protein 5) (Dastur et al. 2006; Wong et al. 2006; Takeuchi et al. 2006) has been shown to be the major E3 for ISG15 conjugation. Like Ub, ISG15 is removed from conjugated proteins by a specific protease, USP18 (Malakhov et al. 2002). All enzymes identified in ISG15 conjugation pathway so far are induced by type I IFNs. UbcH8 was also reported to be a ubiquitin-conjugating enzyme, but it is clear now that UbE1L selectively binds to UbcH8 (Durfee et al. 2008) and favors ISG15 conjugation. In addition to the major enzymes described above, UbcH6 (Takeuchi et al. 2005) and EFP (estrogen-responsive finger protein; also called TRIM25) (Zou and Zhang 2006) are also identified as E2 and E3 for ISG15 conjugation, respectively. (Figure 1.11)

The biological functions of protein ISG15 conjugation in cells remain to be elucidated. At least 158 ISG15 conjugation targets in human cells have been identified so far (Zhao et al. 2005; Giannakopoulos et al. 2005). The identity of these ISG15 target proteins provides opportunities to study the function of ISG15 conjugation. Most target proteins are constitutively expressed and function through diverse cellular pathways, such as RNA splicing, stress response, chromatin remodeling, transcription, and translation. Twelve of the ISG15 target proteins are IFN α/β induced proteins. Nine of these IFN$-\alpha/\beta$ induced proteins can be characterized as antiviral proteins: PKR, MxA, GBP-1, HuP56, HuP54, HuP60, HuP58, RIG-I, and STAT1 (Zhao et al. 2005). One possibility is that ISG15 modification of these IFN$-\alpha/\beta$ induced antiviral proteins increase their stability and/or activity. ISG15 might also change the activities of its target proteins, perhaps by altering their localization or association with other proteins.

Modification by ISG15 does not appear to target proteins for proteasomal degradation. One function of ISG15 might be to stabilize proteins that would otherwise be targeted for Ub-mediated degradation. ISG15 has been reported to prevent virus-

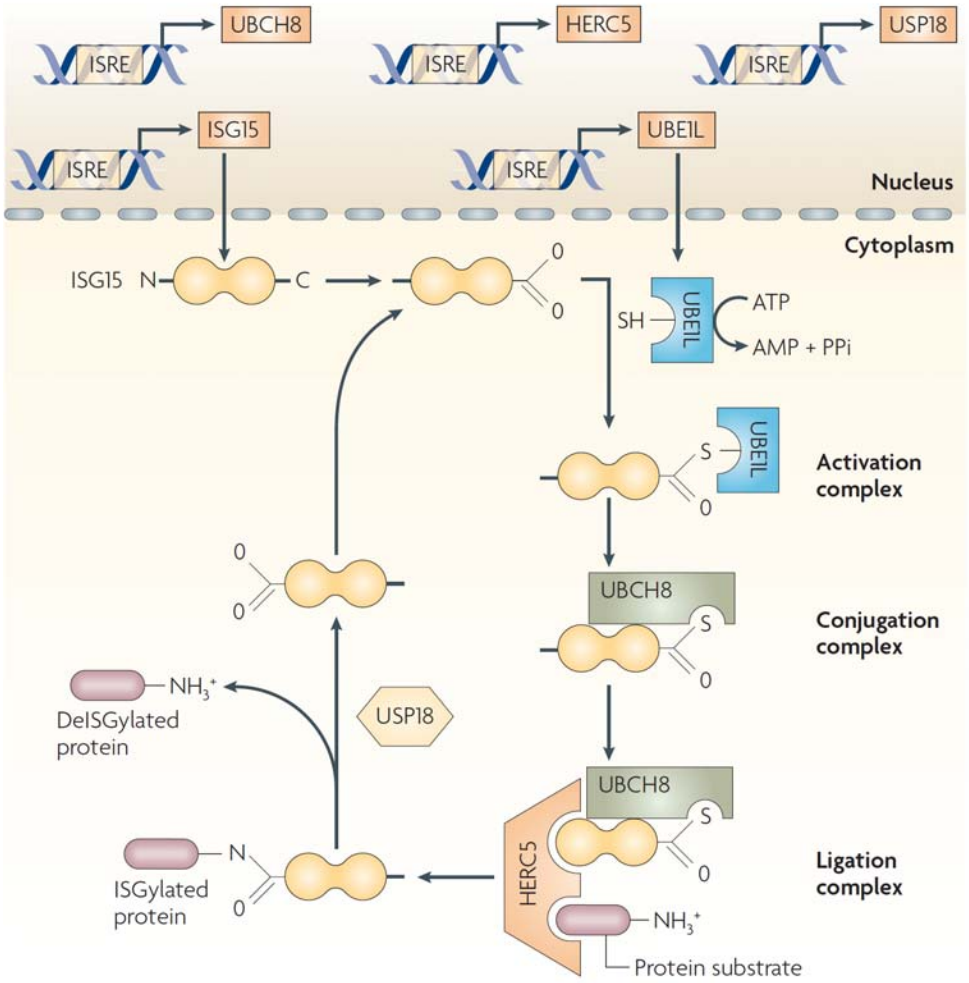


Figure 1.11 Schematic diagram of ISG15 conjugation pathway. See text for details. Adapted from Sadler and Williams (2008).

mediated degradation of IFN-regulatory factor 3 (IRF-3) and increase the induction of IFN-β expression (Lu et al. 2006).

ISG15 and/or its conjugation are proposed to have a role in the innate immune response to viral or bacterial infection. Although ISG15 knockout (ISG15-/-) mice show neither defect in the antiviral response against vesicular stomatitis virus (VSV) and Lymphocytic Choriomeningitis virus (LCMV) nor enhanced STAT1 signaling (Osiak et al. 2005), these mice have increased susceptibility to influenza A and B viruses, Sindbis virus, Herpes simplex virus 1 (HSV-1) and murine γ –herpesvirus. Increased lethality and virus replication in multiple organs during virus infection in ISG15-/- mice provided strong evidence that ISG15 and/or its conjugation have antiviral function (Lenschow et al. 2005; Lenschow et al. 2007). One hypothesis is that ISG15 functions by antagonizing ubiquitin's activity by competing for ubiquitin conjugation sites on specific host or viral proteins. This is supported by recent studies that overexpression of ISG15 suppresses the ubiquitination of viral proteins that is required for efficient virus release, via inhibiting ubiquitination of HIV proteins Gag and Tsg101 (Okumura et al. 2006) or inhibiting the Nedd4 Ub E3 activities in Ebola virus infected cells (Malakhova and Zhang 2008). In the case of HIV-1 and Ebola virus, the antiviral function is most likely mediated by free ISG15 alone (Malakhova and Zhang 2008; Okumura et al. 2006). In contrast, a study of the effect of ISG15 on Sindbis virus replication provided initial evidence that ISG15 conjugation may be responsible for the antiviral activity (Lenschow et al. 2005). Specifically, the C-terminal LRLRGG motif of ISG15, which is required for conjugation, is also required for antiviral activity against Sindbis virus. In addition, infection of influenza B virus strongly induces the expression of free ISG15 protein, but NS1B protein blocks protein ISG15 conjugation through direct interaction with ISG15 at its N-terminus (Yuan et al. 2002; Chang et al. 2008). This finding indicates that ISG15

conjugation is likely to be an important part of the IFN α/β-induced antiviral response. The ISG15 conjugation targets involved in the antiviral activities could be host proteins that either directly affect viral replication or in some way modulate the innate immune response. Alternatively, ISG15 could conjugate to viral proteins and inhibit their functions. Modification of viral proteins by either ubiquitin or UBLs, such as SUMO, has been seen with both RNA and DNA viruses. However, although a large number of cellular proteins are ISG15 conjugated, ISG15 conjugation of a virus-encoded protein has not yet been reported.

Instead, ISG15 antiviral activity may be completely independent of its ability to modify target proteins. Human ISG15 has been reported to be a cytokine. Therefore, in addition to its ability to conjugate to intracellular proteins, ISG15 may also function as a cytokine whose activity could impact upon either the innate or adaptive immune response. But the conjugation and cytokine activity of ISG15 might still be linked, since it has been reported that the cytokine activity of ISG15 requires the terminal diglycine (D'Cunha et al. 1996), and a high molecular mass form of ISG15 is present in the serum of infected mice (Lenschow et al. 2007).

The increased susceptibility to influenza A virus infection of ISG15-/- mice was not observed when mouse embryo fibroblasts derived from these mice were used in tissue culture experiments (Lenschow et al. 2007). These investigators postulated that the analysis of the effects of ISG15 and/or its conjugation on influenza A virus replication had to be carried out *in vivo* in mice. In this thesis, we focus on human tissue culture cells, and demonstrate that IFN-induced ISG15 conjugation inhibits influenza A virus replication in these cells. Consequently, human tissue culture cells provide a tractable system to delineate how ISG15 conjugation inhibits influenza A virus replication.

Chapter 2 : ISG15 conjugation has anti-influenza effect and can be examined in human tissue culture models

2.1 INTRODUCTION

Virus infection activates the synthesis of type I interferons (IFN-α and IFN-β), which induce hundreds of IFN-stimulated genes (ISG), many of which play crucial roles in the antiviral response (Biron and Sen 2001; Randall and Goodbourn 2008; Sadler and Williams 2008). One of the earliest and most strongly induced proteins is ISG15, a 15-kDa ubiquitin-like protein that becomes conjugated to many cellular proteins through its C-terminal LRLRGG motif (Korant et al. 1984; Blomstrom et al. 1986; Haas et al. 1987; Loeb and Haas 1992; Loeb and Haas 1994; Narasimhan et al. 1996; Malakhov et al. 2003; Zhao et al. 2005; Giannakopoulos et al. 2005; Wong et al. 2006; Pattyn et al. 2008). The three enzymes in human cells that catalyze this conjugation, the UbE1L E1 enzyme, the UbcH8 E2 enzyme and the Herc5 E3 ligase, as well as the deconjugating enzyme, UBP43, are also induced by IFN-α/β . (Yuan and Krug 2001; Zhao et al. 2005; Dastur et al. 2006; Wong et al. 2006; Malakhov et al. 2002) Although it had been reported that UbcH8 functions in both ISG15 and ubiquitin conjugation (Kumar et al. 1997; Zhang et al. 2000; Chin et al. 2002; Dastur et al. 2006; Wong et al. 2006), a recent study demonstrated that UbcH8 is unlikely to function in ubiquitin conjugation *in vivo*, based on the high Km of the E1 ubiquitin-activating enzyme for UbcH8 and on the low level of UbcH8 expression in the absence of IFN treatment (Durfee et al. 2008). A large number of human proteins that are targets for ISG15 conjugation have been identified (Zhao et al. 2005; Giannakopoulos et al. 2005; Wong et al. 2006; Pattyn et al. 2008). Most of these targets are constitutively expressed proteins that function in diverse cellular

pathways, but several of the targets are IFN-α/β induced antiviral proteins, including MxA, GBP-1, HuP56 (p56), RIG-I, PKR, HuP54, HuP60, HuP58, and STAT1. The exact function of ISG15 conjugation is still not clear, but ISG15 modification of these IFN–α/β induced antiviral proteins may increase their stability, activity, and/or interaction with other proteins

The rapid induction of ISG15 after viral infection and the existence of ISG15-conjugated antiviral proteins has raised the speculation of ISG15 being an antiviral molecule. Because the NS1 protein of influenza B virus (NS1B) was shown to bind ISG15 and inhibit its conjugation to target proteins, it was proposed that ISG15 and/or its conjugation are inhibitory to the replication of influenza B virus (Yuan and Krug 2001). In addition, ISG15 overexpression was shown to inhibit the release of HIV-1 virions *in vitro* (Okumura et al. 2006). Subsequently, experiments using ISG15-knockout (ISG15-/-) mice established that ISG15 and/or its conjugation inhibits the replication of not only influenza B virus but also influenza A virus (Lenschow et al. 2007). For example, at one of the inoculum levels employed for influenza A virus, 52% of the ISG15-/- mice died, whereas significantly less, 23%, of the ISG15+/+ mice died. However, the effect of ISG15 and/or its conjugation on virus replication was not detected in mouse embryo fibroblasts (MEFs) in tissue culture. MEFs only supported very limited replication of influenza A virus, and there was no significant difference in virus replication between ISG15+/+ and ISG15-/- MEFs (Lenschow et al. 2007). These investigators postulated that influenza A virus replication was probably selectively spared in other, not yet identified cell types of the ISG15-/- mouse and that analysis of the effects of ISG15 and/or its conjugation on influenza A virus replication had to be carried out *in vivo* in mice.

In the present study we focus on human tissue culture cells and on the effect of ISG15 and/or its conjugation on the replication of influenza A virus in such cells. We show that IFN-induced antiviral activity against influenza A virus is significantly alleviated by blocking ISG15 conjugation using small interfering RNAs (siRNAs) against ISG15 conjugating enzymes, thereby demonstrating that IFN-induced ISG15 conjugation inhibits influenza A virus replication in human tissue culture cells. Unconjugated ISG15 does not contribute to this antiviral activity because the siRNA knockdowns had essentially no effect on the level of free ISG15. Finally, we used siRNA knockdowns in human cells to demonstrate that other IFN-induced proteins, specifically p56, MxA and phospholipid scramblase 1, also inhibit influenza A virus gene expression.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines

A549 human lung carcinoma cells (ATCC CCL-185), Vero African green monkey kidney cells (ATCC CCL-81), Madin–Darby canine kidney (MDCK) (ATCC CCL-34), and Calu3 (ATCC HTB-55) were purchased from ATCC. ISG15 wt (ISG15 +/+) and ISG15 knockout (ISG15 -/-) mouse embryo fibroblasts were kindly provided by Dr. Klaus-Peter Knobeloch and Dr. Ivan Horak (Osiak et al. 2005). MEFs, A549, Vero, and MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO®), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO®) at 37°C under a 5% CO_2/95% air atmosphere. Calu3 cells were grown in Advanced MEM (GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum

(FBS), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under a 5% CO_2/95% air atmosphere.

2.2.2 RNA interference.

All siRNA duplexes were synthesized by Invitrogen and resuspended in DEPC-treated water to a final storage concentration of 20μM. siRNA sequences used in the experiment are listed in table 1.1 (only one strand of each siRNA duplex is shown). For ISG15, UbE1L, and UbcH8 RNAi, ISG15 conjugation knockout efficiency of each siRNA duplex was examined by transfecting siRNA into A549 cells followed by 24-hour human IFN-β treatment. Both UbcH8 siRNA duplexes showed the same high efficiency for ISG15 conjugation knockout (more than 90% reduction of UbcH8 protein expression and ISG15 conjugation in western blot analysis). Each of the three ISG15 siRNA duplexes showed similar 50-60% reduction of ISG15 conjugation and relatively low, if not none, reduction of free ISG15 protein level. Each of the three UbE1L siRNA duplexes also showed 50-60% reduction of ISG15 conjugation. However, when applying ISG15 and UbE1L RNAi together, ISG15 conjugation were efficiently reduced to the level comparable to that of UbcH8 knockout experiments, i.e., more than 85-90% reduction in ISG15 conjugation. All nine combinations of ISG15- and UbE1L- siRNA duplexes used in these double knockout experiments had the same extent of effects in reducing ISG15 conjugation. For MxA RNAi, both siRNA duplexes were effective and knocked down about 80% of the IFN-induced MxA protein expression. A pool of all three siRNAs was used to knockout p56, p54, phospholid scramblase 1 (PLSCR1), GBP-1, or viperin.

Table 2.1 Sequences of siRNA used in present study.

siRNA	Sequence (sequence of complimentary strand not shown)
UbcH8 (a)	5’-CCUACCACCUGAAAGCCUUdTdT
UbcH8 (b)	5’-GACGAGAACGGACAGAUUUdTdT
ISG15 (a)	5’-AAUCUUCUGGGUGAUCUGCGCCUUC
ISG15 (b)	5’-AGAGGUUCGUCGCAUUUGUCCACCA
ISG15 (c)	5’-CCCUUGUUAUUCCU-CACCAGGAUGC
UbE1L (a)	5’-UAGUGCUGGCGUCUCAGUUUCUCCU
UbE1L (b)	5’-UAAGGUGAGUGUCUGUGGCUCAUCC
UbE1L (c)	5’-UUCCCGAGAAAGUCACCAAGUCUCC
MxA (a)	5’-GGAAGCUGUGAGAGCAGUUUGGUUU
MxA (b)	5’-GCUUGCUUUCACAGAUGUUUCGAUA
p56 (a)	5’-ACAGAGUCUUGGAUCAGAUUGAAUU
p56 (b)	5’-ACUUAAUGCAGGAAGAACAUGACAA
p56 (c)	5’-GCAACUUUGCCUGGAUGUAUUACCA
Phospholipid scamblase 1 (a)	5’-GGAAACAAACUUGCCAGUUGGGUAU
Phospholipid scamblase 1 (b)	5’-ACUGUCCACCUGGAUUAGAAUAUUU
Phospholipid scamblase 1 (c)	5’-GCAGCGGAAGAUACUGAUUGCUGUA
GBP-1 (a)	5’-UCCUUUCUGCCAUUACACAGCCUAU
GBP-1 (b)	5’-ACGGUGCAGUCUCACACUAAAGGAA
GBP-1 (c)	5’-GAAGCUUGCCCAGCUCGAGAAACUA
Viperin (a)	5’-ACAUAUGGAGU-AAGGCUGAUCUGAA
Viperin (b)	5’-GGUUCCAGAAUUAUGGUGAGUAUUU
Viperin (c)	5’-GGAAGCUCUU-GAGUGUGUUCAGGCA

For obtaining the maximal RNAi effect, confluent A549 cells were trypsinized, washed, and resuspended in serum- and antibiotics-free Opti-MEM at 3×10^5 cells per ml. For Calu3 cells, the cells were resuspended at 6×10^5 cells per ml. 2.5ml cell suspension was seeded into one well of a 6-well tissue culture plate immediately before siRNA transfection. For each siRNA transfection, Xtreme® siRNA transfection reagent (Roche) was diluted with Opti-MEM I (Invitrogen) and incubated at room temperature for 5 minutes. SiRNA was mixed with Opti-MEM I and added to the diluted transfection reagent in a ratio of 2.5μl reagent/20nM siRNA(final concentration after added to cells), and the mixture was incubated at room temperature for additional 25 minutes. The transfection complex was then added to A549 cells. The plate was gently rocked to mix the cells and transfection complex. Final concentration of siRNA in each well was 20nM, except for ISG15 and UbE1L double knockdown, where 40nM of UbE1L and 60nM of ISG15 siRNAs were used. SiRNA-transfected cells were incubated in serum- and antibiotics-free Opti-MEM I (Invitrogen) for 12-24 hours at 37°C. The culture medium was replaced with DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 units/ml penicillin, and 100μg/ml streptomycin for A549 cells, or Advanced MEM with 10%FBS and penicillin-streptomycin-glutamine for Calu3 cells. Where indicated, human interferon β (100-1000u/ml) (Betatheron®, Berlix Co.) was added. Cells were mock- or IFN-β- treated for 24 hours prior to influenza A virus infection.

2.2.3 Virus infection

Recombinant influenza A/Udorn/72 (Ud) virus stocks were amplified in 10-day fertilized eggs, and the virus titers were determined by plaque assays in Madin-Darby

canine kidney (MDCK) cells (described in details in "Methods and Materials" of chapter 3). For single-cycle virus infections, cells (A549, Calu3, or MEFs) were infected with the Ud virus at a high multiplicity of infection (moi) of 5 plaque-forming units (pfu)/cell. In brief, cells were washed twice with PBS to remove remnant serum in the culture medium before infection. Influenza virus was diluted in serum-free DMEM and added to the washed cells at multiplicity of infection (MOI) of 5 pfu/cell. After 1h adsorption at 37°C, cells were washed once with PBS, replenished with infection media, and the cells were incubated at 37°C under 5% CO_2 for indicated time. For protein analysis, the infection medium is composed of DMEM with 2% heat-inactivated FBS and penicillin-streptomycin (A549, Vero, and MEFs) or Opti-MEM I containing 2% FBS and antibiotics (Calu3 cells). For virus analysis in Calu3 cells, infection medium was composed of Opti-MEM I, 2.5µg/ml N-acetylated trypsin, and penicillin-streptomycin. At time-point indicated, supernatants were collected from the culture media of infected cell, and virus titers were determined by plaque assay in MDCK cells.

ISG15+/+ and ISG15-/- MEFs were split into 6-well tissue culture plates. When cells reach 90% confluency, culture media were changed with or without 1000u/ml recombinant mouse interferon beta (Sigma-aldrich, I9032). After 24h interferon treatment, MEFs were infected by influenza A/WSN/33, A/PR8, or A/Udorn/72 virus at a high multiplicity of 5 pfu/cell for 6 hours.

Wild-type vesicular stomatitis virus (VSV) was kindly provided by Dr. Robert H. Silverman (Cleveland Clinic). VSV was propagated, and the titer of the virus stock was measured by plaque assays in Vero cells. In short, viral infections were performed in serum-free DMEM for one hour. After the incubation, infection medium was replaced with DMEM containing 2% FBS and antibiotics for virus propagation, or fully supplemented growth medium for protein analysis. The plaque assay in Vero cells is

modified from plaque assays in MDCK cells. One hour post-infection, virus inoculum was removed and replaced with 1% agarose resuspended in DMEM containing 2%FBS and penicillin-streptomycin. Three days post-infection, agarose was removed and the cells were fixed with 4% formaldehyde in PBS for 30 minutes at room temperature. Cells were then stained with 0.2% crystal violet resuspended in 20% ethyl alcohol, and washed with water. Virus plaques were counted to determine the virus production.

2.2.4 RNA Extraction, Reverse Transcription (RT), Semi-quantitative RT-PCR, and Northern analysis

A549 cells were transfected with either control or ISGylation pathway-specific siRNAs, IFN-treated, and infected with influenza A/Udorn/72 virus for 8 hours. After 8 hours of infection, culture medium was removed, and cells were washed once with PBS. Cells were then lysed, and total RNA was harvested by using Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNAs of vRNA, cRNA, or mRNA of NS gene were produced by employing 1μg of total RNA and 10pmol specific primers for reverse transcription using Transcriptor First Strand cDNA Synthesis Kit (Roche) in a 20μl reaction mixture. 5μl mixture of RT reaction was then mixed with NS gene specific primers and platinum PCR supermix (Invitrogen) for semi-quantitative PCR analysis. PCR were terminated at 20, 25, and 30 cycles. Amplified PCR products were visualized by 1% agarose electrophoresis. For Northern blot analysis, 10μg total RNA/sample was separated on 1.2% agarose gel. RNA was then transferred and UV-crosslinked onto nylon membrane (Nytran®, Whatman Schleicher & Schuell). To prepare the ^{32}P-labeled probes for NS1, the PCR product of full-length NS gene was used as template for the random priming reaction. Random primers were produced by Klenow fragment in the presence of α-^{32}P -dCTP using RadPrime® DNA labeling system (Invitrogen). Unincorporated

nucleotides were removed by using ProbeQuant 50 (GE Healthcare). After hybridization, signal strength was determined by scanning the activated phosphor imaging screen (Bio-Rad) with Typhoon Trio (GE Healthcare) and analyzed by ImageQuant software.

2.2.5 Antibodies and Immunoblotting

Antibodies against the following proteins were used to probe immunoblots: human ISG15; influenza A virus NS1A protein; UbcH8 (Abgent); mouse ISG15 (Osiak et al. 2005); the major structural proteins of influenza A/Udorn/72 virus, which detects the HA, NP and M1 (matrix) proteins, provided by Dr. Robert A. Lamb (Northwestern University); p56, provided by Dr. Ganes Sen (Cleveland Clinic); VSV N protein, provided by Dr. Robert H. Silverman (Cleveland Clinic); MxA, provided by Dr. Otto Haller (Universitätsklinikum Freiburg); and viperin, provided by Dr. Peter Cresswell (Yale University).

Cells collected at the indicated time were extracted in RIPA buffer (50mM Tris-Cl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete® protease inhibitor (Roche). Proteins (30μg/sample) were separated on 12% polyacrylamide-sodium dodecyl sulfate gels (SDS-PAGE) and transferred onto nitrocellulose membrane (Bio-Rad) by semi-dry transfer method at 0.8mA/cm^2. Blots were blocked in TBS containing 0.2% Tween (TBST) and 5% non-fat dried milk at room temperature for 1 h and then incubated with primary antibodies in the same blocking buffer at room temperature for 1 h or at 4°C overnight. The blots were washed three times with TBST. Secondary antibodies, rabbit anti-mouse IgG-HRP (Santa Cruz), donkey anti-rabbit IgG-HRP (GE Amersham), or donkey anti-goat IgG-HRP (Santa Cruz) were diluted (1:10000) in blocking buffer. Membranes were incubated in these diluted secondary antibodies for an hour at room temperature and washed four

times with TBST. Proteins were detected with enhanced chemiluminescence (ECL) western blotting substrate (Pierce) and exposed to CL-Xposure film (Pierce).

2.3 Results

2.3.1 SiRNA knockdown of ISG15 conjugation alleviates the IFN-induced inhibition of influenza A virus gene expression

The basic experimental design is diagrammed on the top of Figure 2.1. Human A549 cells were treated with a high level of IFN-β (1000 units/ml) for 24 hours to achieve sufficient ISG15 conjugation. As a control, a different set of cells was not treated with IFN. Twenty-four hours prior to IFN treatment, the cells were transfected with siRNA(s) directed against the enzyme(s) in the ISG15 conjugation pathway to selectively suppress IFN-induced ISG15 conjugation. As a control, another set of cells was treated with scrambled control siRNA. After the successive siRNA and IFN treatments, the cells were infected with influenza A/Udorn/72 (Ud) virus at a high multiplicity of infection (moi) 5 pfu/cell for 8 hours, and the synthesis of viral proteins and RNAs was measured by immunoblots (WB) and northern blots (NB) as described below.

We first targeted UbE1L, the E1 enzyme in ISG15 conjugation (Yuan and Robert M. Krug 2001). However, siRNA knockdown of UbE1L in A549 cells alone did not achieve efficient inhibition of ISG15 conjugation, and it was necessary to add a siRNA directed against ISG15 itself. As analyzed by an immunoblot, the combination of these two siRNAs effectively eliminated IFN-induced ISG15 conjugation (Figure 2.1 (A), compare lanes 3 and 4). In contrast, only a minimal reduction in free ISG15 occurred. Consequently, it was not clear why it was necessary to add the siRNA directed against ISG15 to achieve efficient inhibition of ISG15 conjugation. Other experiments in which we used siRNAs against only ISG15 also failed to effectively reduce the amount of IFN-

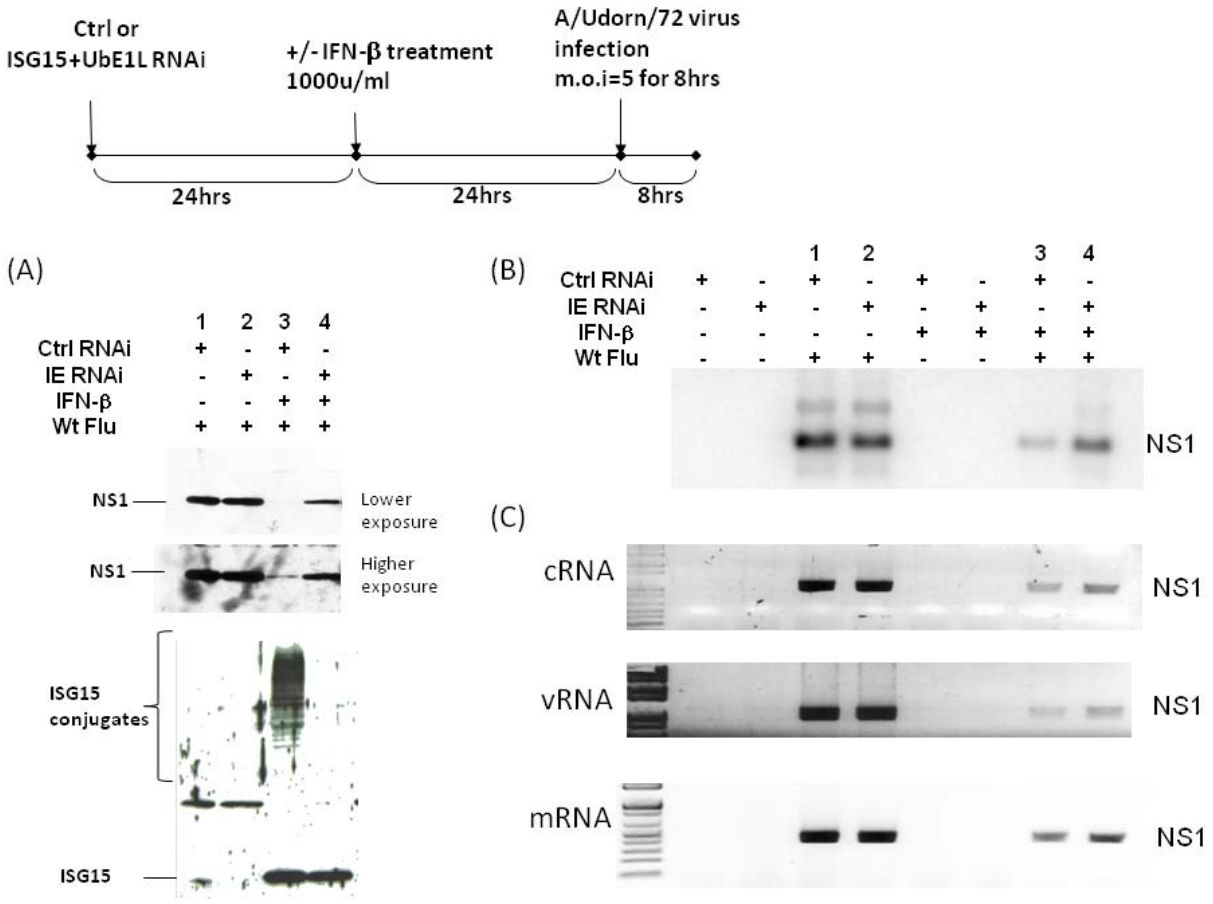


Figure 2.1 Knocking down ISG15 conjugation by RNAi alleviates the inhibition on viral gene expression induced by IFN-β. A549 cells treated with siRNAs and IFN-β, as shown in the schematic diagram of experimental design, were infected by influenza A/Udorn/72 viruses at m.o.i. of 5 pfu/cell for 8 hours. (A) Western blot analysis showed that ISG15 conjugation induced by IFN was knocked down by combined treatment of ISG15 and UbE1L siRNAs. Influenza viral protein expression (NS1) was partially rescued in these knocked down cells. (B) Northern blot analysis and (C) semi-quantitative RT-PCR analysis indicated that increased viral RNA synthesis were also presented in these ISGylation-knocked down cells.

induced ISG15 (data not shown). By knocking down ISG15 conjugation with the UbE1L and ISG15 siRNAs, the synthesis of the viral NS1A protein was partially rescued (Figure 2.1(A)). The amount of the NS1A protein in the cells transfected with these two siRNAs (lane 4) was approximately 10-fold higher than in the cells transfected with the control siRNA (lane 3). The amount of the restored NS1A protein was approximately 15% of the amount produced in infected cells not treated with IFN (compare lane 4 to lanes 1 and 2). A similar restoration of the synthesis of NS1A RNA was observed in the cells treated with the two specific siRNAs, as assayed by Northern blot analysis (Fig. 2.1 (B)). Semi-quantitative RT-PCR analysis confirmed the northern blot result and further revealed that it was a combined effect of increases in all 3 RNA species, cDNA, vRNA, and mRNA (Fig. 2.1(C)). Consequently, we concluded that ISG15 conjugation inhibits the synthesis of both the NS1A protein and its viral RNAs.

To verify that ISG15 conjugation rather than free ISG15 mediated this inhibition of NS1A protein synthesis, we used an siRNA that targeted UbcH8 (Figure 2.2) (Zhao et al. 2004; Dastur et al. 2006; Wong et al. 2006; Durfee et al. 2008). The UbcH8 siRNAs effectively inhibited ISG15 conjugation, whereas no effect on the accumulation of free ISG15 was detected (Figures 2.2(A)). The effect of the UbcH8 siRNA on NS1A protein synthesis was similar to that obtained in the experiment using the UbE1L plus ISG15 siRNAs (Figures 2.2(B)): (i) the amount of the NS1A protein in the cells transfected with the UbcH8 siRNA (lane 4) was 10-20-fold higher than in the cells transfected with the control siRNA (lane 3); and (ii) the amount of rescued NS1A protein level was 15-20% of that in cells not treated with IFN (compare lane 4 to lanes 1 and 2). These results verify that ISG15 conjugation rather than free ISG15 inhibits the synthesis of the influenza A virus NS1A protein in human cells.

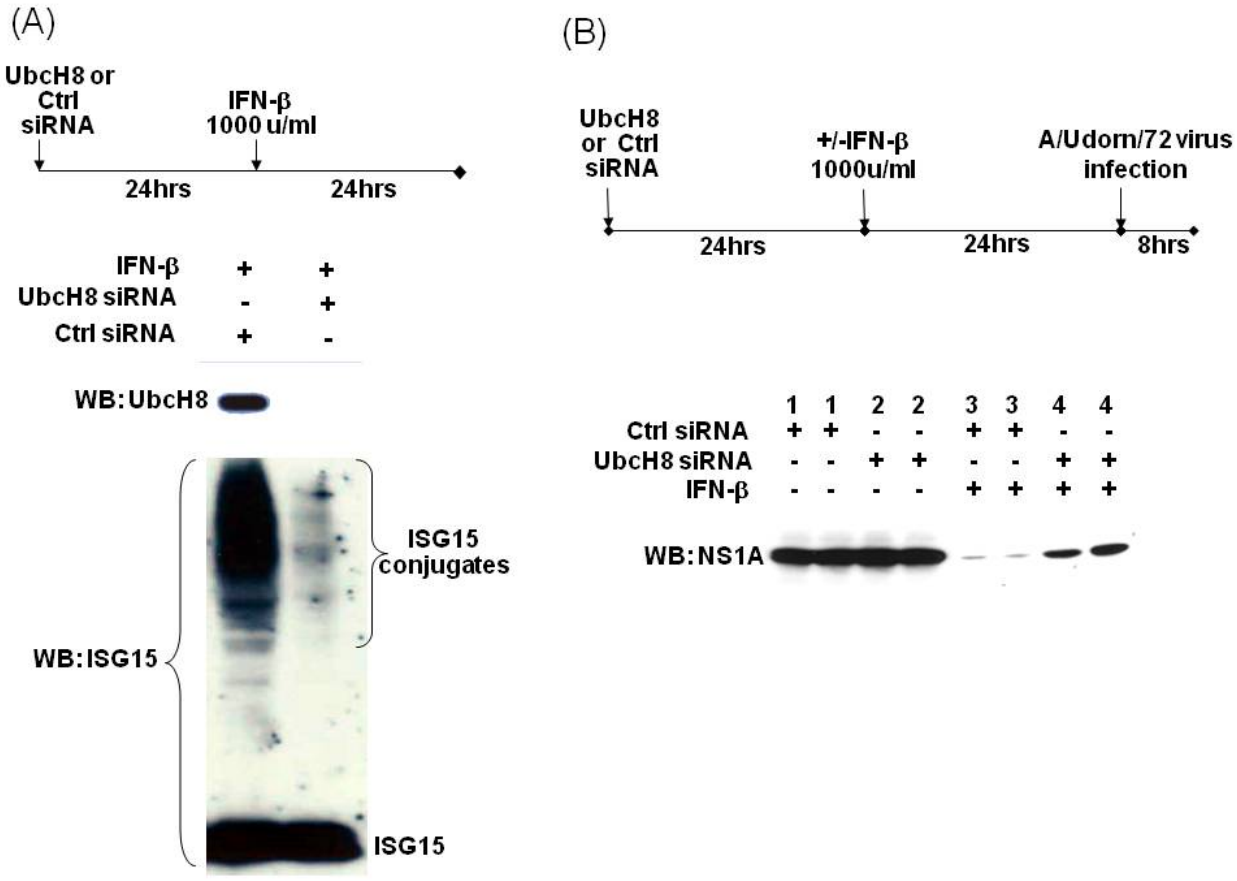


Figure 2.2 Eliminating ISGylation by knocking down UbcH8 also relieves the inhibition of IFN-β on influenza viral protein expression. (A) ISG15 conjugation in A549 cells induced by IFN-β treatment was knocked out by UbcH8 siRNA treatment. (B) UbcH8 siRNA and IFN-β treated A549 cells were infected by influenza A/Udorn/72 viruses. Inhibition on viral protein expression by IFN treatment was partially relieved in these cells.

To determine whether ISG15 conjugation similarly inhibits the synthesis of other influenza A virus proteins, we measured the production of the major viral structural proteins using an antibody raised against Ud virions (Figure 2.3). Knocking down ISG15 conjugation with the siRNAs directed against UbE1L and ISG15 rescued the synthesis of not only the NS1A protein but also the HA, NP and M1 structural proteins. Similar to the situation with the NS1A protein, the amount of these three structural proteins in cells transfected with the UbE1L and ISG15 siRNAs (lane 4) was approximately 20-fold higher than in the cells transfected with the control siRNA (lane 3); and the rescued HA, NP and M1 protein levels were 15-20% of the levels in cells not treated with IFN (compare lane 4 to lanes 1 and 2). Consequently, ISG15 conjugation inhibited the synthesis of most, if not all influenza A virus-encoded proteins.

In the study of characterizing the ISG15 antiviral activity in ISG15-/- mice, these mice are more susceptible to infection of influenza, sindbis, and herpex simplex virus type I, but no defects were found in their responses to vesicular stomatitis virus (VSV) or lymphocytic choriomeningitis virus (LCMV). We decided to examine if ISG15 conjugation had antiviral effect against VSV infection in human tissue culture cells as seen with influenza virus infection. ISG15 conjugation in A549 cells were knocked down by RNAi as previously described. The cells were then IFN-treated for 24 hours prior to VSV infection at a high multiplicity of 5 pfu/cell. VSV N protein expression was heavily inhibited in IFN-treated cells (Figure 2.4. Compare lanes 6 and 8 to lanes 1 and 2). But, unlike influenza A virus infection, no significant increase of the protein expression of VSV N protein was found in cells where ISG15 conjugation is knocked down by UbcH8 RNAi (Figure 2.4. Compare lanes 6 and 8). This result was consistent with the findings that ISG15 -/- mice was resistant to VSV infection.

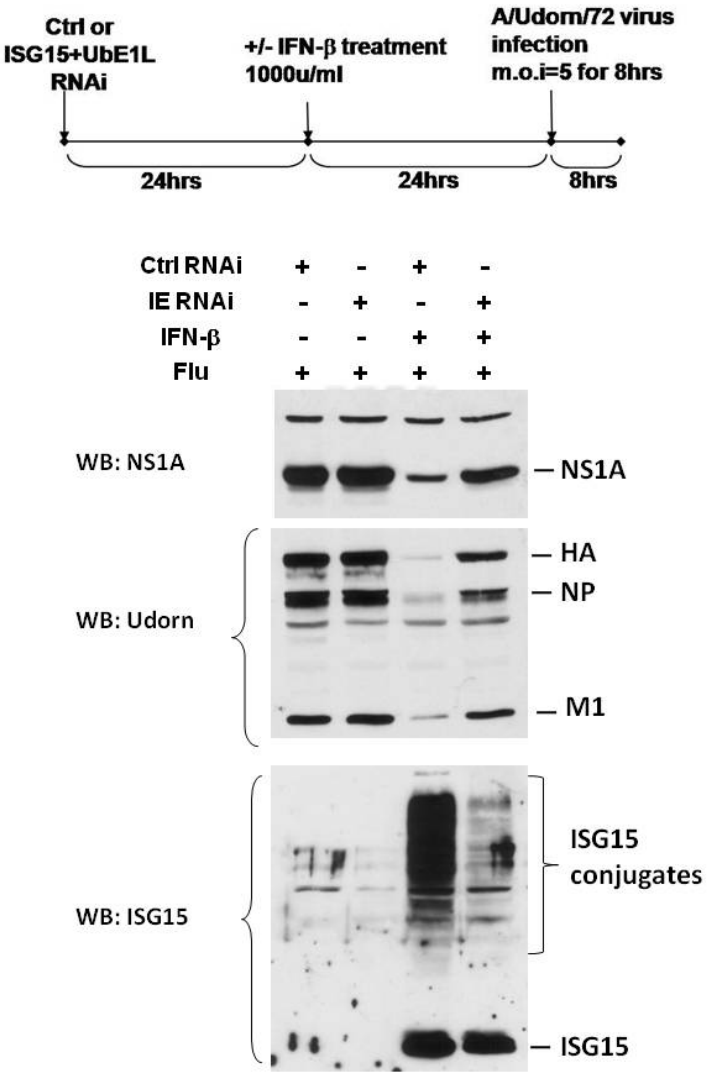


Figure 2.3 Eliminating ISGylation in IFN-β treated cells rescues the protein expression of most viral proteins of influenza A virus. ISG15 conjugation in A549 cells induced by IFN-β treatment was knocked out by ISG15 plus UbE1L siRNAs. Viral protein expressions were analyzed by western blot using specific antibodies.

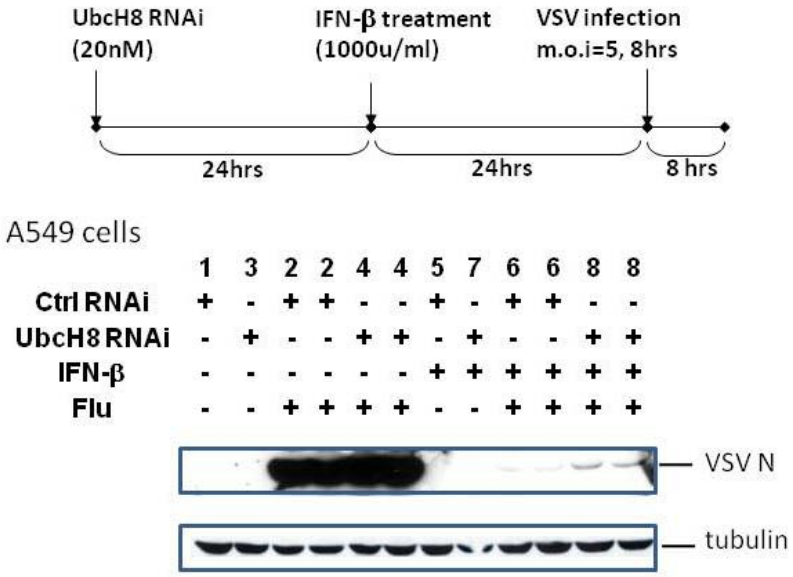


Figure 2.4 Knocking down ISG15 conjugation in IFN-β treated A549 cells does not relieve the inhibition on VSV viral protein expression. A549 cells were siRNA and IFN-β treated as indicated in the schematic diagram. Cells were then infected by VSV at MOI of 5 pfu/cell for 8 hours. Viral protein expression was examined by western blot analysis using antibody against the N protein of VSV.

2.3.2 The inhibition of influenza A virus gene expression by ISG15 conjugation in human cells is sufficient to result in inhibition of virus replication

To determine whether ISG15 conjugation inhibits the replication of influenza A virus in human cells, we used Calu3 cells which afford high replication rates of influenza A virus (Zeng et al. 2007). We measured the production of progeny virus in IFN-treated Calu3 cells that had been transfected either with the control siRNA or with both the UbE1L and ISG15 siRNAs (Figure 2.5). Transfection of Calu3 cells with the control siRNA in the absence of IFN treatment induced some ISG15 conjugation (Figure 2.5 A, lane 1), thereby presumably potentiating subsequent IFN-induced ISG15 conjugation (lane 3). Nonetheless, the UbE1L and ISG15 siRNAs inhibited ISG15 conjugation in Calu3 cells approximately 90% (lane 4), and the amount of the NS1A protein synthesized in cells transfected by these two siRNAs was 10-15-fold higher than in cells transfected with the control siRNA (compare lanes 3 and 4). The effect on virus replication was similar: the rate of virus replication during the first 4 hours of infection in cells transfected with these two siRNAs was approximately 10-20 fold greater than in cells transfected with the control siRNA (Figure 2.5B). We conclude that ISG15 conjugation in human cells inhibits influenza A virus replication.

2.3.3 The anti-influenza activities of ISG15 conjugation in mouse and human cells are different.

ISG15-/- mice had higher susceptibility to influenza A viruse infection than wild-type mice, and the virus titer was also higher in the lungs of these infected ISG15-/- mice. However, the differences of virus production were not detected when ISG15 +/+ and ISG15 -/- MEFs were used; viral growth of influenza A virus was limited in both MEFs (Lenschow et al. 2007). We examined the influenza A virus gene expression in these MEFs in a setting similar to that used in the siRNA experiment.

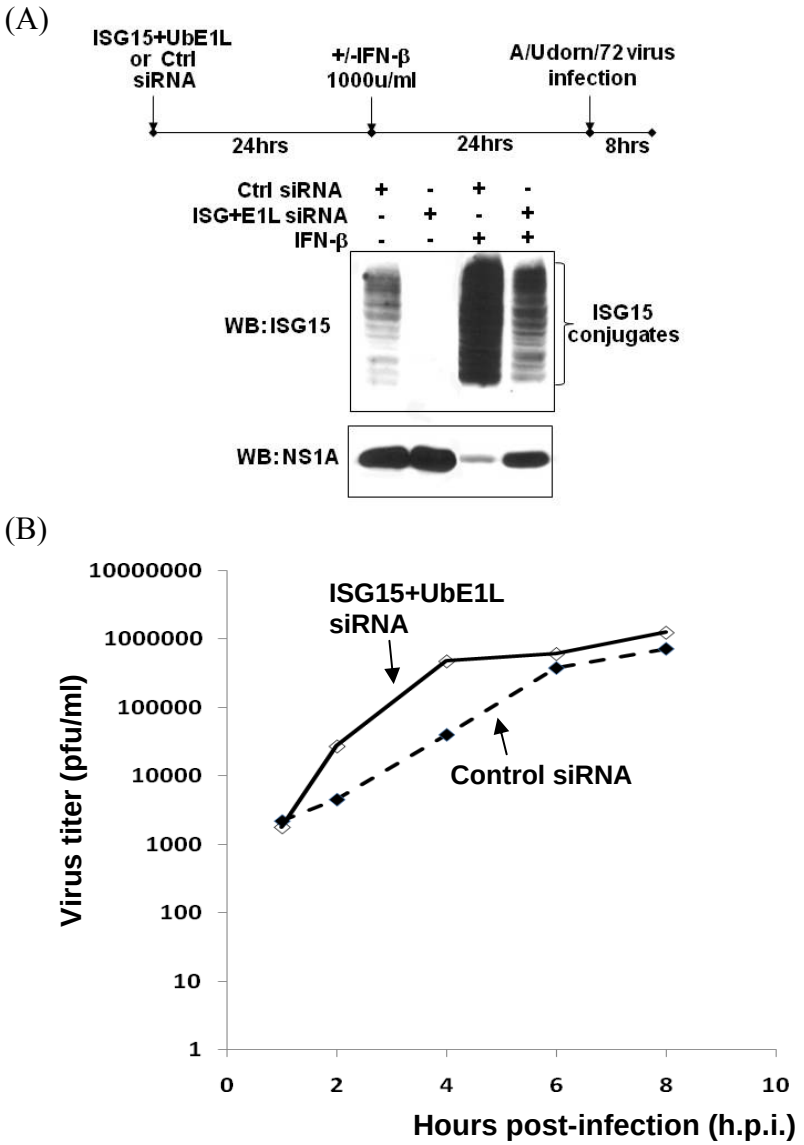


Figure 2.5 The inhibition of influenza A virus gene expression by ISG15 conjugation in human cells is sufficient to result in inhibition of virus replication. ISG15 conjugation induced by IFN-β treatment in Calu3 cells were knocked down by RNAi as described previously. Cells were subsequently infected by influenza A/Udorn/72 virus for 8 hours. Supernatants from infected cells were collected every two hours post-infection for plaque assay analysis. (A) Cell lysates were harvested 8 hours after infection for western blot analysis (B) Virus production from each time point was measured by plaque assays in MDCK cells.

ISG15+/+ and ISG15-/- MEFs were treated with or without mouse IFN-β for 24 hours, and infected with A/WSN/33, A/PR8, or A/Udorn/72 influenza viruses at high multiplicity for 6 hours. ISG15 conjugation was greatly induced in ISG15+/+ MEFs, but was not seen in ISG15-/- MEFs. Notably, in ISG15+/+ MEFs without interferon treatment, mild level of ISG15 conjugation was detected. Influenza NS1A protein was expressed at high level in non-IFN treated MEFs; the mild ISGylation detected in ISG15+/+ MEFs did not affect the protein synthesis. In IFN-treated MEFs, the NS1A protein expression was heavily inhibited, and there was no significant difference of the NS1A protein expression between ISG15 +/+ and ISG15 -/- MEFs (Figure 2.6). This result is consistent with the previous findings that viral replication was not increased in infected ISG15 +/+ or ISG15 -/- MEFs. However, in our study, we have shown that in human tissue culture cells, ISG15 conjugation knockdown is correlated with increased influenza viral protein expression and virus production. These findings indicate that the MEFs are not the suitable mouse cell lines to study the function of ISG15 conjugation, or, the anti-influenza activity in mouse and human might function through different mechanisms. The latter hypothesis is more preferable as research in our lab indicates that NS1 protein of influenza B virus specifically binds to human ISG15, but not to mouse ISG15 (data not shown), suggesting a specific role of ISG15 conjugation in antiviral activity in human cells. Unlike ubiquitin, which is highly conserved (>95%) among different species, human and mouse ISG15 proteins only has 66.7% homology (NCBI HomoloGene).

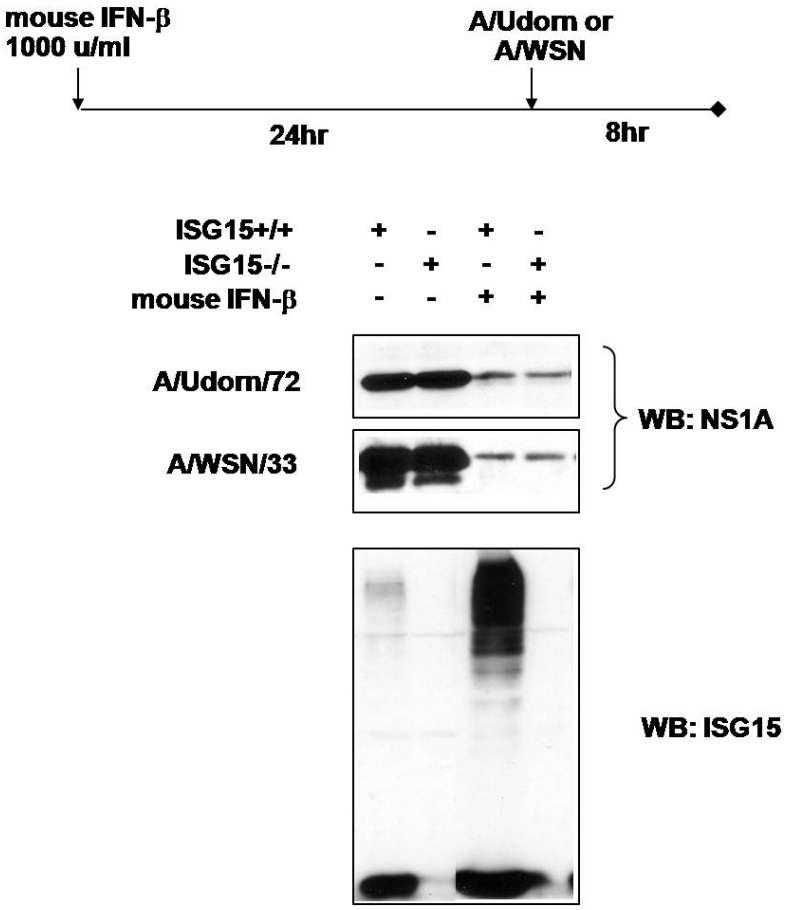


Figure 2.6 Influenza viral protein expression was not rescued in IFN-β treated ISG-/- MEFs. ISG15+/+ and ISG15 -/- MEFs were either mock-treated or IFN-treated for 24 hours. Cells were then infected with either A/WSN/33 or A/Udorn/72 viruses. Protein expression of NS1A and ISG15 conjugation were detected by western blot analysis.

2.3.4 SiRNA knockdown of several other IFN-induced proteins alleviates the IFN-induced inhibition of influenza A virus gene expression in human cells

Our observation that siRNA knockdown of IFN-induced ISG15 conjugation only partially relieves the antiviral effect of IFN treatment on influenza A virus gene expression and replication in human cells indicates that, as to be expected, other IFN-induced proteins also contribute to the IFN-induced inhibition of influenza A virus replication. To identify at least some of these antiviral proteins, we transfected siRNAs directed against each of several IFN-induced proteins with known or suspected antiviral activities against other viruses (Figure 2.7). We used the same basic experimental design as employed for the siRNA knockdowns of IFN-induced ISG15 conjugation. SiRNAs against p56, MxA and phospholipid scramblase 1 (PLSCR1) rescued the synthesis of the viral NS1A protein to essentially the same extent as the siRNA knockdown of ISG15 conjugation, demonstrating that these three IFN-induced proteins inhibit influenza A virus gene expression. In contrast, rescue was not detected using the siRNA directed against GBP-1, but since we did not have antibody against the GBP-1 protein, we could not determine whether GBP-1 expression was efficiently reduced by the siRNA. The siRNA against viperin, which efficiently reduced the amount of this protein, caused at most a marginal rescue of the synthesis of the NS1A protein; consistent with the evidence that viperin predominantly inhibits release of the virus from the plasma membrane, a step that occurs after most viral protein synthesis (Wang et al. 2007). The minimal effect caused by viperin siRNA serves as an additional negative control for these siRNA knockdown experiments, verifying that the rescue of influenza A virus gene expression caused by siRNAs directed against other IFN-induced proteins demonstrates that such IFN-induced proteins contribute to the IFN-induced antiviral activity against influenza A virus.

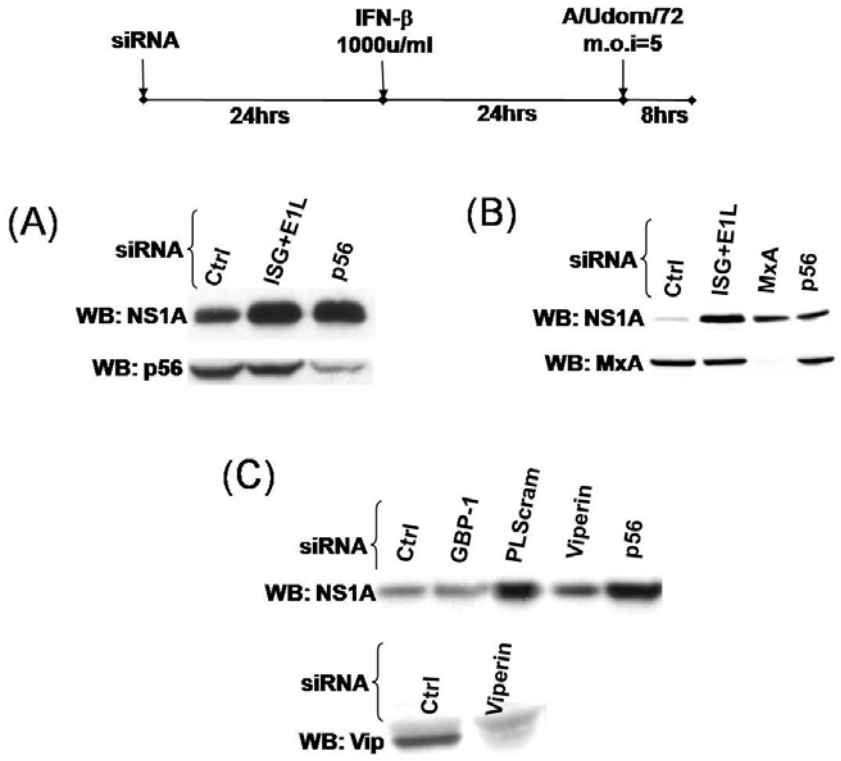


Figure 2.7 HuP56, MxA and phospholipid scramblase 1 RNAi also rescued influenza viral protein expression inhibited by IFN-β treatment. A549 cells were transfected with different antiviral siRNAs and treated with IFN-β for 24 hours. Cells were subsequently infected with influenza A/Udorn/72 viruses. Viral protein expression (NS1) was examined to reveal the rescue effects of knocking down these antiviral proteins. (A) Effect of p56 knockdown was compared to that of the ISGylation knockdown experiment. (B) MxA knockdown was compared with ISGylation knockdown and p56 knockdown experiments. (C)Effects of GBP-1, PLSCR1, and viperin knockdown experiments were compared to that of the p56 knockdown experiment.

2.4 DISCUSSION

IFN-α/β treatment of human cells induces the synthesis of ISG15 and three of its conjugating enzymes, resulting in the conjugation of a large number of proteins (Korant et al. 1984; Blomstrom et al. 1986; Haas et al. 1987; Loeb and Haas 1994; Narasimhan et al. 1996; Yuan and Krug 2001; Zhao et al. 2004; Dastur et al. 2006; Wong et al. 2006; Durfee et al. 2008; Zhao et al. 2005; Pattyn et al. 2008). Experiments using ISG15-knockout (ISG15-/-) mice established that ISG15 and/or its conjugation inhibits the replication of influenza A virus. (Lenschow et al. 2007). In the present study we demonstrated that IFN-induced ISG15 conjugation inhibits influenza A virus gene expression and replication in human tissue culture cells. By siRNA-mediated depletion of the two enzymes that catalyze ISG15 conjugation, we demonstrated that suppression of ISG15 conjugation in human cells caused approximately a 10-20-fold reduction in the IFN-induced antiviral activity against influenza A virus gene expression and replication. Based on the amount of restoration of influenza A virus gene expression by these ISGylation pathway-specific siRNAs, we estimated that ISG15 conjugation comprises approximately 15% of the IFN-induced antiviral activity against influenza A virus in human cells. Free ISG15 does not contribute to this antiviral activity against influenza A virus because the siRNA knockdowns had little or no effect on the level of free ISG15, but these experiments do not rule out the possibility that free ISG15 may have additional antiviral activity against influenza A virus in human cells. Our results establish that ISG15 modification of target proteins inhibits influenza A virus gene expression and replication in human tissue culture cells. In contrast, a significant effect of ISG15 and/or its conjugation on influenza A virus infection has not been detected in at least one mouse cell line, specifically MEFs. A previous study reported there was no significant difference

in influenza A virus replication between ISG15+/+ and ISG15-/- MEFs (Lenschow et al. 2007). We confirmed these results, and also found that pretreatment of these MEFs with mouse IFN-β to induce efficient ISG15 conjugation resulted in the same extremely efficient inhibition of influenza A viral gene expression in both ISG15+/+ and ISG15-/- MEFs. These results suggest that there are important differences between the mouse and human ISG15 conjugation systems with regard to their antiviral activities against influenza A virus. In fact, it is already known that human ISG15 shares only 66.7% homology with mouse ISG15.

Recent studies indicated that ISG15 has antiviral activities against other viruses. In the case of HIV-1 and Ebola virus, the antiviral function is most likely mediated by free ISG15 alone, which suppresses the ubiquitination of viral proteins that is required for efficient virus release (Malakhova and Zhang 2008; Okumura et al. 2006). In contrast, a study of the effect of ISG15 on Sindbis virus replication provided initial evidence that ISG15 conjugation may be responsible for antiviral activity (Lenschow et al. 2005). Specifically, the C-terminal LRLRGG motif of ISG15, which is required for conjugation, is also required for antiviral activity against Sindbis virus. Here we provide direct evidence that ISG15 modification of target proteins inhibits influenza A virus gene expression and replication in human tissue culture cells. ISG15 conjugation could exert its antiviral activity against influenza A virus by two mechanisms that may not be mutually exclusive. In one mechanism, ISG15 conjugation of cellular target proteins, e.g., the previously identified antiviral MxA and p56 protein targets, is required for, or at least strongly enhances, their antiviral activities. In the second mechanism, ISG15 conjugation of the viral protein(s) inhibits one or more of its essential functions. Based on the present study, human cells in tissue culture should provide tractable systems to delineate the mechanisms by which ISG15 conjugation inhibits influenza A virus

replication. Our lab is currently investigating possible ISG15 conjugation of influenza viral proteins and identifying the conjugation site(s). Once the site(s) is determined, recombinant virus expressing mutant protein(s) resisting ISG15 modifications can be generated and assayed in human cells by the RNAi approach described in this study to determine the functional relevance of such ISG15 conjugation on viral proteins. If the ISG15 conjugation of the viral protein inhibits its functions, the mutant viruses without the conjugation site could escape from the ISG15 antiviral effects, thus showing better replication after IFN pretreatment and less sensitivity to ISG15 siRNA knockdown.

Recent studies have identified there are at least two ISG15 E3 enzymes, namely Herc5 and Efp. Interestingly, the two enzymes have different target specificity: Efp only targets 14-3-3 protein, while Herc5 can function on all the targets assayed in co-transfection experiments. In our future study, we will also use the RNAi protocol to examine the role of Efp and Herc5 in the antiviral activity of ISG15 conjugation, which could help us to clarify that which of these ISG15 conjugation targets is important for its antiviral activity. The siRNA knockdown experiments also demonstrated that three other IFN-induced proteins, MxA, p56 and phospholipid scramblase 1, inhibit influenza A virus replication in human cells. Our MxA results confirm previous experiments which showed that constitutive expression of MxA in the absence of IFN inhibits influenza A virus replication (Pavlovic et al. 1992). In contrast, p56 and phospholipid scramblase 1 had not been shown previously to inhibit influenza A virus replication, although they had been shown to inhibit the replication of other RNA viruses (Dong et al. 2004; Zhang et al. 2007; Wang et al. 2003). Consequently, the present study and those of others have demonstrated that the IFN-induced antiviral activity against influenza A virus in human cells is mediated at least in part by ISG15 conjugation, MxA, p56, phospholipid scramblase 1, and viperin (Pavlovic et al. 1992; Wang et al. 2007; Lenschow et al. 2007).

In contrast, the 2'-5' oligo(A)synthetase/RNase L pathway and PKR do not inhibit influenza A virus replication because the viral NS1A protein blocks their inhibitory activity (Min and Krug 2006; Min et al. 2007).

So far, all of the RNAi experiments presented in this study target single antiviral protein and result in only partial relief from IFN inhibitory effects. In the future, the combination of siRNAs target different potential antiviral proteins will be employed in search of the maximal synergetic effects. Such investigation could provide further insights into the mechanistic aspects about the action of individual antiviral protein, especially the possible crosstalk between these molecules. In this respect, it would be of special interest to determine whether knocking down ISG15 and its target proteins together would promote further influenza viral gene expression than single ISGylation knockdown experiment.

Chapter 3 : Identify NS1A interacting proteins during viral infection in vivo

3.1 INTRODUCTION

The non-structural NS1 protein of influenza A virus (NS1A) is an important multifunctional virulence factor (Horimoto and Kawaoka 2005; Noah and Robert M. Krug 2005); it counteracts host cell antiviral defenses by engaging in multiple protein-RNA and protein-protein interactions (Krug et al. 2003). NS1A protein is composed of two domains: the N-terminal 73 amino acids comprise the RNA-binding domain which forms a symmetric homodimer with a unique six-helical chain fold (Liu et al. 1997; Chien et al. 1997; Yin et al. 2007); the remaining amino acids constitute the effector domain. The RNA-binding domain binds dsRNA with low affinity, but is functionally implicated in protecting influenza A virus against the antiviral state induced by IFN-β through inhibiting the IFN-α/β-induced 2'-5'-oligo (A) synthetase (OAS)/RNase L pathway (Min and Krug, 2006). The effector domain fulfilled multiple biological functions by binding to an array of the cellular proteins: (i) the inhibition of the 3'- end processing machinery by binding to the 30-kDa subunit of the cellular cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II (PABII) (Nemeroff et al. 1998; Chen et al. 1999; Li et al. 2001; Noah et al. 2003; Twu et al. 2006;); (ii) inhibition of the activation of dsRNA-activated protein kinase (PKR)by directing binding to PKR(Li et al. 2006; Min et al. 2007); and (iii) the activation of phosphatidylinositol-3-kinase (PI3K) signaling by binding to p85β subunit of PI3K (Hale et al. 2006; Shin et al. 2007a; Ehrhardt et al. 2007). In addition, one of the most recent

study found that NS1A from avian strains, but not from human strains, interacts with CrkL/CrkL in virus-infected cells (Heikkinen et al. 2008). The biological relevance of this strain-specific interaction is still unknown. It is also suggested that NS1A interacts with other influenza viral proteins, e.g. NP and influenza viral polymerases, in viral infected cells (Marion et al. 1997). However, the direct binding between NS1A and NP or polymerases could not be established *in vitro* and further study is required to fully understand how these interactions occur during virus infection.

Besides the above NS1A interactions with verified significances in the context of viral infection, there were also a number of other cellular proteins identified previously as NS1A interactors. These proteins include NS1-I (Wolff et al. 1996), NS1-BP (Wolff, et al. 1998), hStaufen (Falcon et al. 1999), eukaryotic translation initiation factor 4GI (eIF4GI) (Aragon et al. 2000), poly(A) binding protein I (PABP1) (Burgui et al. 2003), retinoic acid-inducible gene I product (RIG-I) (Mibayashi et al. 2007), mRNA export machinery (Satterly et al. 2007), and importins (Melen et al. 2007). Although some of these interactions show biological effect in artificial context, e.g. transient co-transfection assay, they lack supporting evidences from direct study of interactions in viral infected cells.

One of the major reasons accounting for that most of previously identified NS1A interacting proteins have not shown biological relevance is the intrinsic limitation in the methods employed for their identification. There have been two major methods in previous search of NS1A interactors: (i) yeast two hybrid screening (ii) co-immunoprecipitation approach. Although these methods are widely used to study protein-protein interactions, they have important disadvantages. With yeast two-hybrid screening, the disadvantage is that the result largely depends on the quality of library used for screening, and it also has limitation in detecting interactions of a protein complex where

strong association is assembled by weak individual protein interactions. For the co-immunoprecipitation approach, anti-serum was used for pulling down NS1A protein from lysates derived from cells where NS1A was either expressed by transfection (most of the cases) or by infection with wild type influenza A virus. The efficiency and specificity of the antibodies greatly affects the outcome. In addition, if specific NS1A-mediated protein interactions, e.g. interaction between NS1A and viral polymerase as discussed further below, are only present in infected cells, these interactions will not be detected by expression of NS1A alone as in the transfection approach. These setbacks restrict previous study to identify the actual catalogue of NS1A interactions during viral infection. To overcome the drawbacks associated with previous approaches studying NS1A-mediated protein interactions, in the present study, we set out to generate a recombinant influenza A virus encoding epitope tagged NS1A protein, which can be used for specific and efficient proteomic analysis in identifying NS1A associated proteins during viral infection.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines

A549 human lung carcinoma cells, Madin–Darby canine kidney (MDCK), and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM)(GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum (FBS)(GIBCO®), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin(GIBCO®) at 37°C under a 5% CO_2/95% air atmosphere. Anti-Py antibody-secreting AK6967 hybridoma cells, provided by Dr, Scott Stevens, were maintained in Dulbecco's modified Eagle's medium (DMEM)(GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum

(FBS)(GIBCO®), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin(GIBCO®) at 37°C under a 5% CO_2/95% air atmosphere as suspended cells in flasks. To harvest antibodies, hybridoma cells were transferred into Protein-free Hybridoma Medium (PFHM) (GIBCO®) without antibiotics or supplements and grown until 3 days past confluence. Cell debris was removed by centrifugation. Supernatant containing antibodies was filtered and saved at -20°C with 0.02% sodium azide.

3.2.2 Generating N-terminal tagged influenza A/Udorn/72 virus

pHH21 plasmids containing the full-length cDNAs for each of the eight influenza A/Udorn/72 genomic RNA segments, and four pcDNA plasmids expressing NP, PA, PB1, and PB2 proteins of influenza A/Udorn/72 were provided by Makoto Takeda (Dr. Robert A. Lamb's lab, Northwestern University). Virus was generated by co-transfecting 293T cells with these 12 plasmids in serum- and antibiotics-free Opti-MEM I medium (GIBCO®). At 12h post-transfection, the media was changed to Opti-MEM I supplemented with 2.5μg/ml N-acetylated-trypsin (Sigma) and penicillin-streptomycin. Seven to eight hours after media change, the 293 T cells were overlaid onto MDCK cells for viral amplification. Culture supernatants were collected and used for HA assay to determine whether substantial amount of virus particles were produced. Clones of infectious recombinant virus were isolated as individual plaques by performing plaque assays in MDCK cells. Serial 10-fold dilutions of virus samples were used for infecting a monolayer of MDCK cells at 37°C. After 1 hour incubation, inoculum was removed, and 1% semisolid agarose resuspended in DMEM (GIBCO®) containing 2.5μg/ml N-acetylated trypsin (Sigma) and antibiotics was overlaid onto the cells. Two to three days after infection, individual plaque was picked, and the agarose plug was stored in 1.2 ml

DMEM containing 1% BSA at -80°C. The isolated plaque was used to further amplify the virus in the allantoic cavity of 10-day-old embryonated chicken eggs (Charles River Laboratories) at 34°C. Allantoic fluid was harvested 48 hours after virus inoculation, and virus aliquots were stored at -80°C. Virus titer (plaque forming units (PFU) /ml) was measured by plaque assays in MDCK cells. Plaques were visualized and counted by staining the cells with either crystal violet or naphethelene blue-black. Viral genomic RNA was extracted by using QIAamp Viral RNA Mini Kit (Qiagen) and subjected to RT-PCR using SuperScript® First-Strand Synthesis system (Invitrogen). The resulting cDNAs of viral genomic RNA segments were sequenced for validation.

To generate epitope-tagged NS proteins, sequence of either 3XFlag tag or 2X polyoma (Py) tag was inserted between 5'UTR and coding sequence of NS gene in pHH21 plasmid. For 2XPy tag, two rounds of PCR reactions were used to insert the 42-nucleotides (2X MEYMPME) into the NS gene. For 3X Flag tag, the entire 69-nucleotides sequence of the tag (MDYKDHDGDYKDHDIDYKDDDDK) was cloned from p3XFlag-CMV10 expression vector (Sigma), and then fused with NS gene by two rounds of PCR. The resulting PCR products containing specific tags with Esp3I sites at both ends, were digested by restriction enzyme, and inserted into pHH21 plasmids. The pHH21-NS plasmids containing epitope tags were sequenced and used in the co-transfecting experiment to produce recombinant viruses as previously described. Viral genomic RNAs of egg-derived recombinant viruses were extracted and reverse-transcribed. cDNAs of these vRNAs were amplified and sequenced for validation of proper introduction of the epitope tag sequences.

3.2.3 Virus and infection

Wild-type Influenza A/Udorn/72 virus encoding either N-terminal 3XFlag or 2XPy tagged NS proteins was referred to as 3Fwt or 2Pwt virus, respectively. For affinity purification, A549 cells were seeded onto 150mm plates and used for infection when cells reached 95-100% confluency. Cells were washed twice with PBS and then either infected with non-tagged wt or tagged virus (3Fwt or 2Pwt) at a multiplicity of 5 pfu per cell. One hour after adsorption in serum-free DMEM (GIBCO®) at 37°C, virus inoculum was replaced by DMEM supplemented with 2% heat-inactivated FBS (GIBCO®) and penicillin-streptomycin (GIBCO®), and incubated at 37°C /5%CO_2 for additional 7 hours before harvesting cell lysates. For multiple cycle growth curve, confluent monolayer of MDCK cells were washed twice with PBS, and influenza viruses were diluted with serum-free DMEM and added to the washed cells for virus adsorption at a multiplicity of infection (MOI) of 0.001 pfu/cell. After incubated at 37°C for 1h, virus inoculum was replaced by serum-free DMEM supplemented with 2.5µg/ml N-acetylated trypsin (Sigma) and penicillin-streptomycin. Supernatants from culture media containing released virus particles were collected every 12 hours for 60 hours, and virus titers were measured by performing plaque assays in MDCK cells. All experiments were done in triplicates, and virus titers were averaged and used to plot growth curve in logarithmic scale.

3.2.4 Affinity purification

Eight hours post-infection, A549 cells were lysed in Tris-buffered Saline (TBS; 50mM Tris-Cl, pH 8.0, 150mM NaCl) plus 1% NP-40 supplemented with Complete® protease inhibitor (Roche) and PhoSTOP® phosphatase inhibitor (Roche). After incubating on ice for 15 minutes, cell lysate was obtained by removing insoluble debris by centrifugation at 14,000rpm for 30 minutes at 4°C. Protein concentration was

measured by Bradford assay. Equal amount of protein from each sample was used for affinity purification. Anti-Flag M2 agarose (Sigma) was used for anti-3XFlag protein purification. For anti-Py affinity purification, anti-Py antibodies harvested from hybridoma cell culture supernatant were either coupled onto protein-G sepharose beads by dimethyl pimelimidate cross-linking, or used with protein-G sepharose as direct co-immunoprecipitation without chemical crosslinking Affinity matrices were washed twice with ice-cold PBS and equilibrated with lysis buffer. Cell lysate was then added to the washed beads and incubated overnight at 4°C for optimal binding. After binding, beads were washed extensively for six times with lysis buffer. For anti-Py purification, the last three washes were performed with modified Buffer D250 (20mM HEPES, pH 7.9, 250mM NaCl, 10mM β-mercaptoethanol, and 8% glycerol). The affinity matrix was equilibrated with elution buffer without peptide after wash. Elution was carried out using 1:1 (v/v) ratio of agarose and 500ng/µl peptide in elution buffer (for anti-3XFlag purification, 50mM Tris-Cl, pH7.5, 150mM NaCl, 0.012% Triton X-100, 10% glycerol, and 0.2mM EDTA; for anti-Py purification, Buffer D250). 3XFlag peptide was purchased from Sigma; Py peptide (EYMPME) was synthesized by Invitrogen. Eluates were concentrated by Microcon YM-10 (Millipore). Purified proteins were separated by 12% SDS-PAGE and visualized by either silver stain (Invitrogen) or colloidal blue staining (Invitrogen) according to manufacturer's protocols.

3.2.5 Immunoblots

Proteins were resolved on 12% SDS-PAGE gel, and transferred onto nitrocellulose membrane (Bio-Rad) by semi-dry transfer method at 0.8mA/cm^2. Membrane containing transferred proteins was blocked in 5% milk in TBST at room temperature for 1h and preceded to western blot analysis. Either rabbit anti-GST-NS1A

or mouse anti-Flag M2 antibodies were used as primary antibody. For rabbit anti-GST-NS1A antibody, it was used at 1:2000 dilution in 5% milk/TBST. To detect 3XFlag-tagged protein, mouse anti-Flag M2 antibody (Sigma) was used at 1:1000 dilution in 5%milk/TBST. Secondary antibody of donkey anti-rabbit HRP was purchase from GE Amersham. Rabbit anti-mouse-HRP secondary antibody was purchased from Santa Cruz. Both secondary antibodies were used at 1:10000 dilution in 5%milk/TBST. Signal was detected by incubating the membrane with Enhanced Chemiluminescent (ECL) western blotting substrate (Pierce) and exposed to CL-Xposure film (Pierce).

3.2.6 Immunofluorescence

A549 cells were seeded onto 4-well chamber slide. While reaching 50% confluency, cells were infected with either wild-type or 3XFlag-tagged influenza A virus at MOI of 5 pfu/cell. After 1h adsorption, virus inoculum was replaced by DMEM supplemented with 2% FBS and penicillin-streptomycin. Six hours post-infection, cells were rinsed with ice-cold PBS and fixed by 4% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were permeabilized with 0.5% Triton X-100, and blocked with 5% normal goat serum in PBS/0.2% gelatin/ 0.1% Tween-20. Cells were then incubated with rabbit anti-GST-NS1A antibody diluted 1:200 in blocking buffer for 1 hour at room-temperature and washed four times with PBS plus 0.1% Tween-20 (PBST), and incubated with Fluorescein isothiocyanate-conjugated (FITC) AffiniPure Goat Anti-Rabbit IgG (Jackson Lab) diluted 1:100 in blocking buffer for 1h at room temperature. After washed 3 times with PBST and one time with PBS, the intracellular localization of NS1A protein was visualized by using fluorescence microscope.

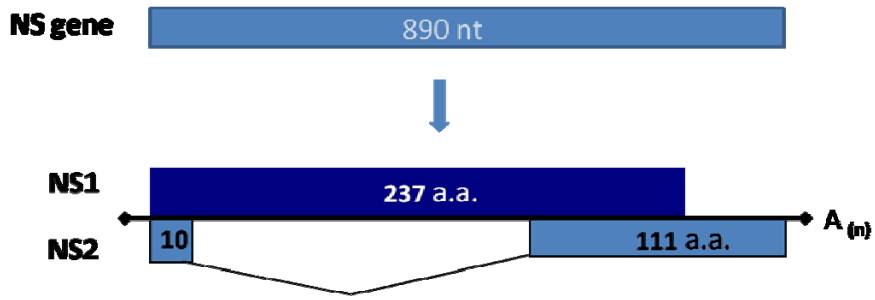
3.3 RESULTS

3.3.1 A recombinant influenza A/Udorn/72 virus containing N-terminal tag NS gene is viable and not attenuated.

The initial purpose of creating influenza A virus with tagged NS1A is to study the protein-protein interaction of NS1A with cellular protein during viral infection. Until now, most NS1A-protein interactions were identified based on the *in vitro* pulled down experiments. However, although some interacting proteins can be readily pulled down *in vitro*, their biological significance was still not clear when the binding mutants were generated, and the interaction did not necessarily reflect the actual events occurring in infected cells. Nevertheless, sometimes when antibodies against specific proteins were used in co-immunoprecipitation experiments to identify the interaction, the non-specific cross-reactivity of the antibodies to other cellular proteins might complicate the interpretation of the existence for direct-binding. Also, while antiserum was used, if the binding sites were pre-occupied by interacting proteins, some interactions might not be detected in the immunoprecipitation results. Therefore, we decided to create influenza A virus containing epitope-tagged NS1A protein, in which the NS1A can be specifically immunoprecipitated against the epitope without interfering with the protein itself. The virus would also be useful to track down the intracellular events during viral infection with its epitope tag.

The influenza NS gene encodes two proteins: NS1, and the spliced form, NS2/NEP (Lamb and Choppin 1979; Lamb and Lai 1980). It would be ideal if only NS1A is tagged with the epitope. However, when the tag was introduced at the 3'end of NS1A ORF, it is inevitable that the NS2 structure will be affected (Figure 3.1(A)).

(A)



(B)

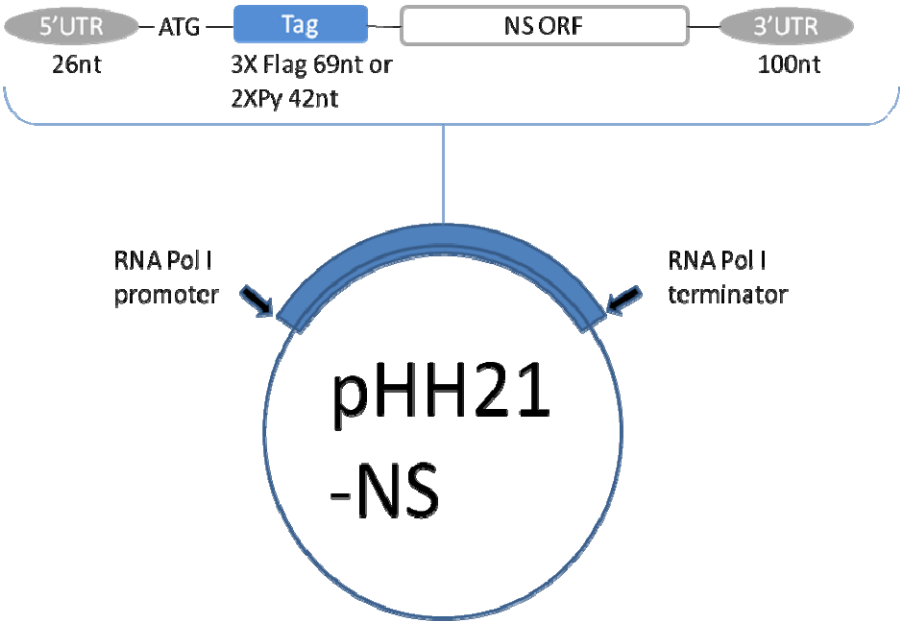


Figure 3.1 **NS gene of Influenza A virus.** (A)NS gene of influenza A virus encodes two proteins, NS1A and NS2A. Alternative splicing of NS mRNA which leads to the production of NS2A protein was shown. (B) Schematic diagram of creating N-terminal epitope-tagged NS construct.

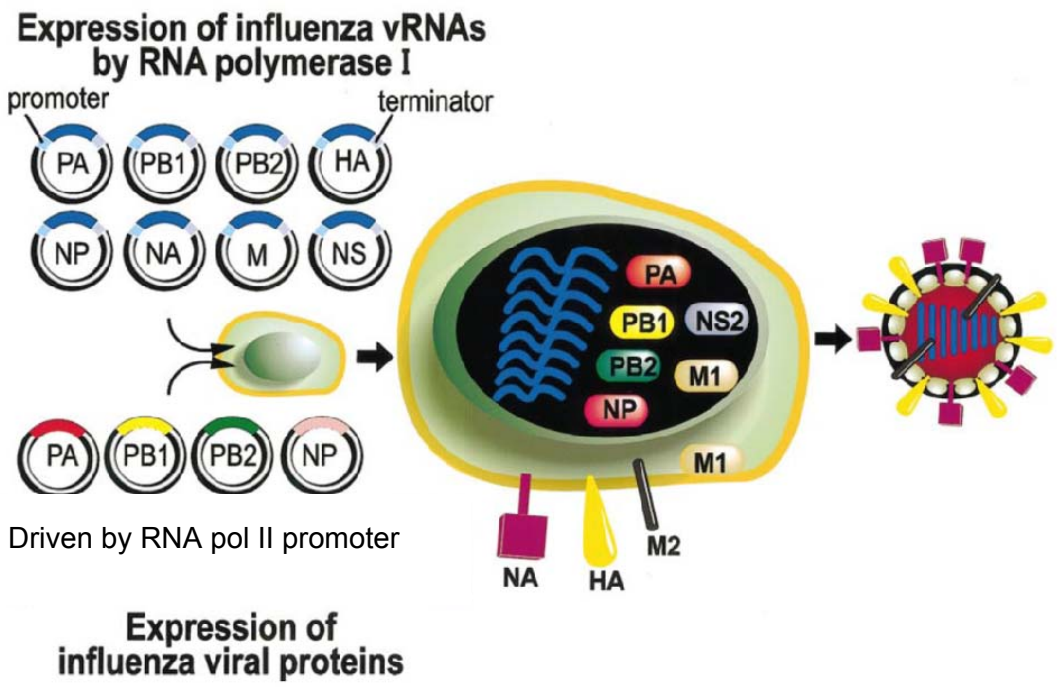


Figure 3.2 Schematic diagram of the strategy of generating recombinant influenza A/Udorn/72 viruses by 12-plasmid co-transfection system. Adapted from Neumann and Kawaoka 2001.

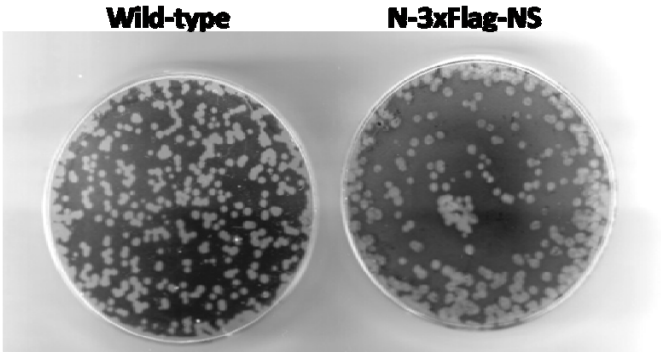
As a result, recombinant virus encoding C-terminal tagged NS1A was not viable. The 69 nucleotides encoding 3XFlag tag or 42 nucleotides encoding 2XPy tag were inserted into NS gene between 5'UTR and start codon of NS open reading frame (Figure 3.1(B)).The resulting DNA was cloned into pHH21 vector to express viral genomic RNA with the tag. The pHH21 construct was then used in creating the recombinant virus in the 12-plasmid transfection system (Figure 3.2).

Both wild-type and tagged viruses were detected in the culture supernatant 5 days after transfection. The virus was further amplified in eggs. Sequencing results confirmed that the tag sequence was included correctly in the NS genomic RNA. The tagged recombinant viruses formed plaques with size the same as wild type in MDCK cells (Figure 3.3(A)), indicating that this virus, which containing N-terminal epitope-tagged NS proteins, is viable and does not have substantial defects compared to wild-type viruses.

To confirm that the tagged NS1A protein is expressed at comparable level as non-tagged wild type virus during viral infection, we infected MDCK cells with both wild-type and 3XFlag-tagged influenza A virus at MOI of 5 pfu/cell, and harvested cell lysates 6 hours post-infection. NS proteins were detected on immunoblots with anti-GST-NS1A or anti-3XFlag antibodies. As shown in Figure 3.3(B), both wild-type and tagged viruses expressed NS1A about the same level. Notably, when anti-Flag antibody was used, NS2A protein was detected in 3XFlag-tagged virus infected cell lysate.

To determine whether the tagged virus has the same growth kinetics compared to non-tagged virus, multiple cycle growth curve in MDCK cells was performed to measure the rate of replication, using a multiplicity of infection at 0.001 plaque-forming units per cell (pfu/cell). The amount of virus produced at each time point was determined by plaque assays in MDCK cells (Figure 3.4).

(A)



(B)

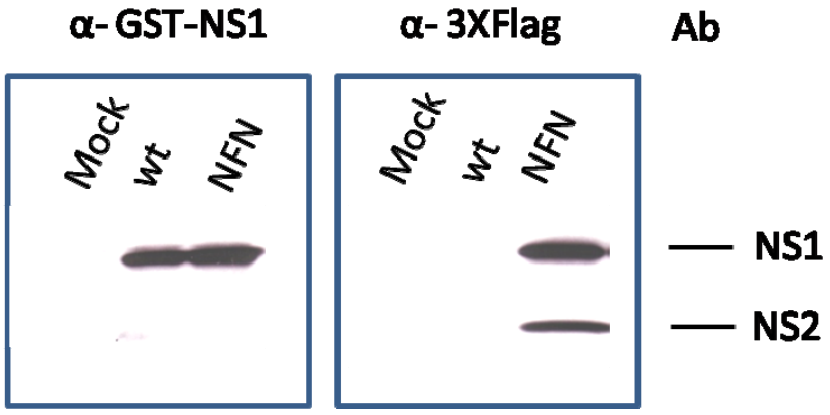


Figure 3.3 The Influenza A/Udorn/72 virus encoding N-terminal epitope tagged NS proteins is not attenuated. (A) Plaques size comparison of wild-type and N-3XFlag NS viruses in MDCK cells. (B)MDCK cells were mock-infected or infected with either wild-type (wt) virus or virus encoding N-terminal 3XFlag tagged NS proteins (NFN). Expression of NS proteins in infected cells was analyzed by western blot. 3XFlag tagged NS1A was detected by either α-GST-NS1 or α-3XFlag antibodies. Non-tagged NS1A was only detected by α-GST-NS1 antibody. NS2A protein was also identified when α-3XFlag antibody was used.

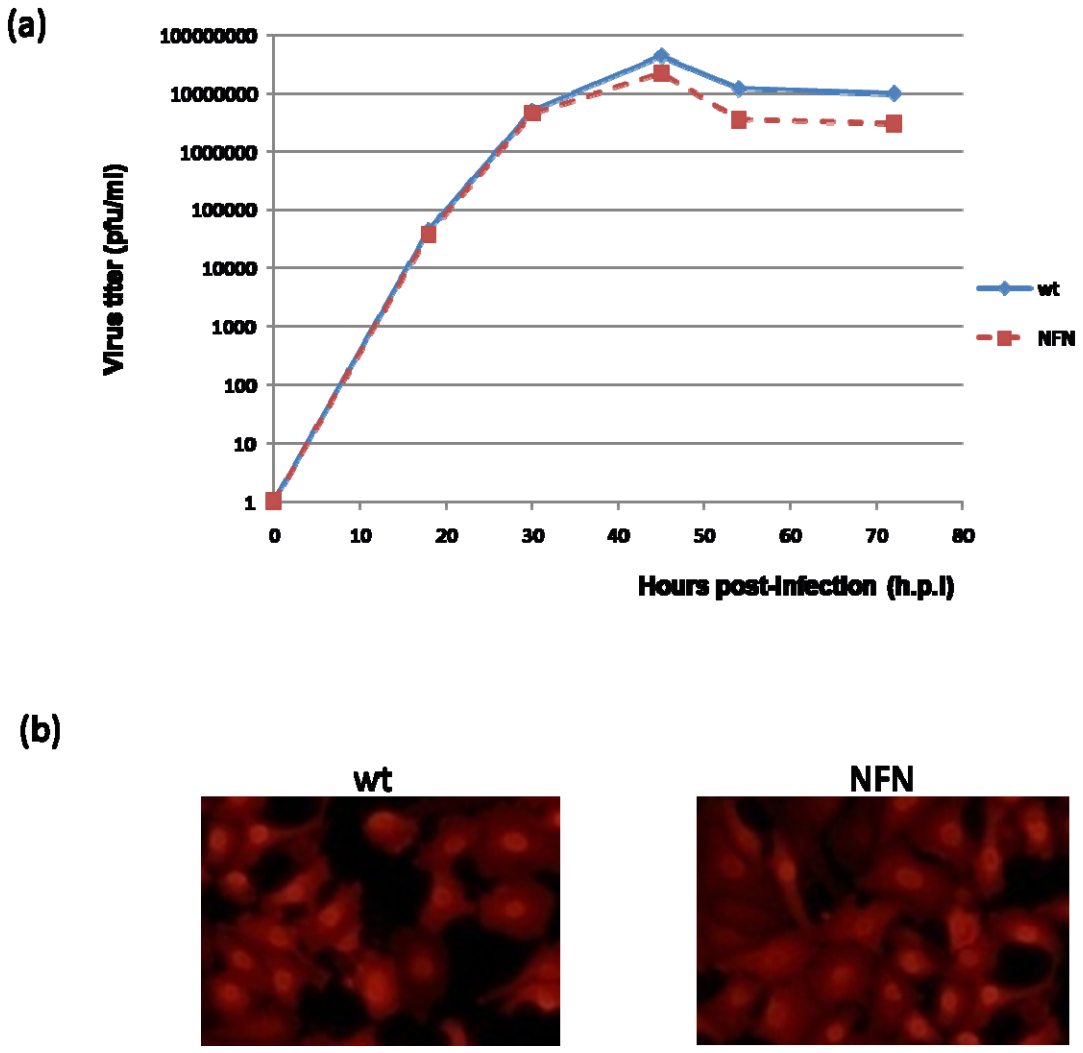


Figure 3.4 Influenza A/Udorn/72 viruses encoding N-terminal 3XFlag tagged NS proteins (NFN) have the same growth kinetics and nuclear localization as non-tagged wild type viruses. (a) Multiple cycle growth curve of non-tagged wild-type and N-3Xflag-NS influenza A/Udorn/72 viruses. MDCK cells were infected at a MOI of 0.001 pfu/cell. Virus production was measured by plaque assays in MDCK cells. (b) Immunofluorescence analysis of the intracellular localization of NS proteins. A549 cells were infected at MOI of 5 pfu /cell. NS proteins were detected by using α-GST-NS1 as primary antibodies for immunofluorescence analysis.

As shown in Figure 3.4(A), both tagged and non-tagged viruses grew at the same rate. Immunofluorescence was carried out to confirm that 3XFlag tagged NS1A also localized in the nucleus as seen in non-tagged wild-type (Figure 3.4(B)). These results indicated N-terminal tagged NS proteins do not interfere with their normal functions, and the tagged viruses behave the same as non-tagged wild-type viruses.

3.3.2 Affinity purification of proteins associated with 3XFlag or 2XPy tagged NS proteins

To pull down NS1/NS2 associated proteins during viral infection, human lung carcinoma A549 cells were infected with influenza A/Udorn/72 viruses encoding either non-tagged or epitope-tagged (3XFlag or 2XPy) NS proteins, at a multiplicity of infection of 5 pfu/cell for 6 hours. Cell lysates were prepared in 1%NP-40 lysis buffer and run through 25 3/8G needle several times. Same amount of total protein from each sample was used for affinity purification. For 3XFlag tagged NS proteins, anti-Flag M2 agarose(Sigma) was used for the purification. For 2XPy tagged NS proteins, two methods were employed to achieve the purpose. Cell culture supernatant of hybridoma cells secreting anti-polyoma (Py) antibodies were collected and incubated with protein-G sepharose beads. These beads were either chemically crosslinked to create affinity column by coupling the antibodies onto beads or were used directly for immuno-precipitation of Py-tagged NS proteins. Significant amount of tagged NS1A proteins was decreased from the input after binding, as shown in Figure 3.5.

After binding, affinity matrices were extensively washed by lysis buffer. Tagged NS proteins and their associated proteins were specifically eluted by 3XFlag or 2XPy peptides.

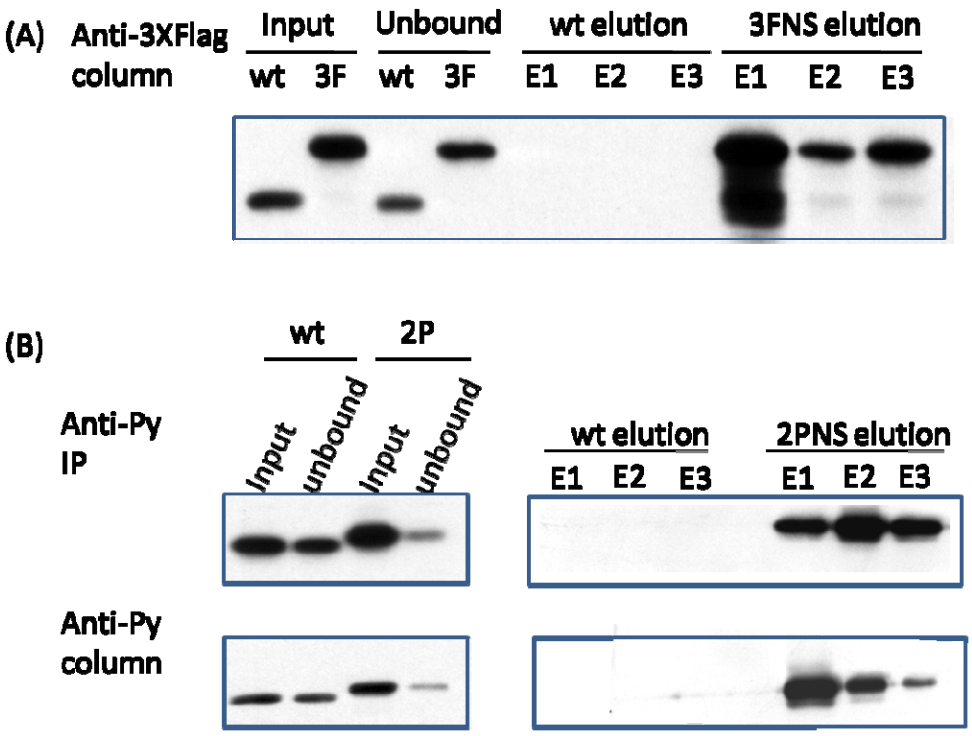


Figure 3.5 **N-terminal tagged NS1 can be efficiently pulled down from infected cell lysate.** A549 cells were infected by wild-type (wt) virus or viruses encoding N-terminal tagged NS proteins: (A) 3XFlag tagged (3F) and (B) 2XPy tagged (2P). After 6 h infection, cell lysates were harvested and followed by affinity purification against specific tags. For 2P viruses, either cross-linked Py column (anti-Py column) or direct immunoprecipitation (anti-Py IP) were used. Bound proteins were eluted by using specific peptide, 3XFlag peptide or 2XPy peptide. Cell lysates before (Input) and after binding (unbound) to the affinity matrices and eluates were analyzed by western blot using antibody against NS1A protein.

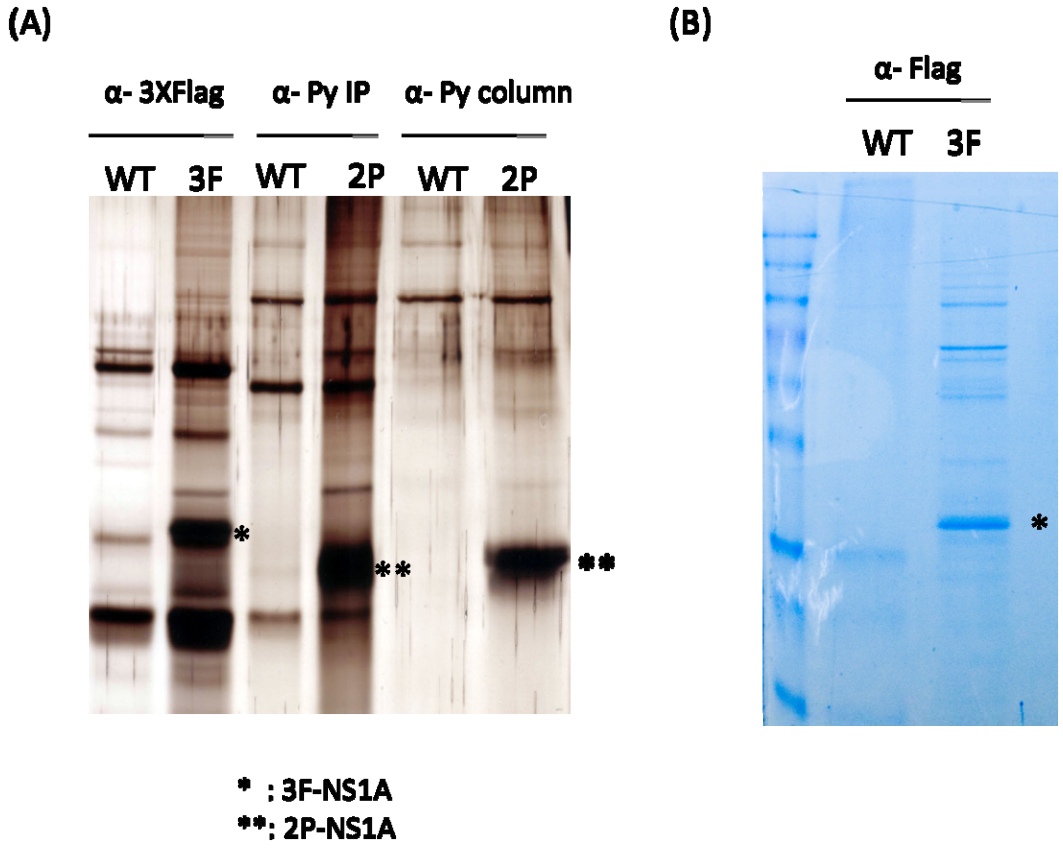


Figure 3.6 Affinity-purified NS1A and interacting proteins were separated by 10% SDS-PAGE. Peptide eluates from affinity purification were resolved on 10% SDS-PAGE. (A) Silver stain; (B) Colloidal Blue stain. wt: wild-type; 3F: 3XFlag; 2P:2XPy. Position of NS1A proteins were marked as indicated.

Elution was carried out several times with small quantities of concentrated peptides. The eluates were resolved on 12% SDS-PAGE gel, and analyzed by western blot, silver stain, or colloidal blue stain as shown in Figure 3.5 and Figure 3.6.

As expected, tagged NS proteins bound to the affinity matrices, and after peptide elution, could be detected with their associated proteins on stained gel. When analyzed by western blot using α-GST-NS1A antibody, signal could only be detected with eluates containing tagged proteins. Non-tagged NS proteins did not bind to the affinity matrix, thus only several background protein bands appeared on the stained gel, and no signal was detected in western blot analysis. This result indicated that influenza A virus with tagged NS gene can be readily used for proteomic study to specifically pull-down protein complexes associated with NS proteins during viral infection, which allows further study of the biological significance of the interactions *in vivo*.

3.4 DISCUSSION

The initial objective of this project is to generate recombinant influenza A/Udorn/72 viruses encoding epitope-tagged NS1A in order to identify its associated proteins during viral infection. In this study, we generate two viruses in which NS1A and NS2A proteins are tagged with either 3XFlag or 2XPy epitope. These two tagged viruses are not attenuated in terms of plaque size or virus replication when compared to the wild-type virus. In addition, the majority of these tagged NS1A proteins, like wild type non-tagged NS1A protein, localize in the nucleus in infected cells. These data collectively demonstrate that the N-terminal epitope tagging does not affect NS1A functions. The advantage of using epitope–tagged bait proteins for identifying their interactors is that they can be readily purified via strong binding to the affinity matrices followed by selective elution with competitive peptide. This approach largely solves the problems

associated with using protein-specific antiserum for direct co-immunoprecipitation, including ineffective antigen-antibody binding and immunoglobin contamination in immunoprecipitates eluted by directly boiling in SDS-sample buffer. Indeed, the results shown in this chapter indicate that the viruses expressing tagged NS1 proteins can be used to effectively purify NS proteins-containing protein complex from infected cells.

However, a new obstacle was encountered during the application of this novel approach, that is, the unwanted tagging of other viral protein. The NS gene produces not only NS1A protein, but also NS2A protein as a result of alternative splicing. These two proteins share the same 10 amino acids at the very N-terminus, as well as the N-terminal epitope in the tagged virus that we generated.

In order to tag only NS1A protein, we should target its C-terminus. However, as discussed earlier, such tagging would greatly alter NS2A coding sequence. NS2A is important for nuclear export of newly synthesized viral proteins and essential for viral replication. Thus, attempts of tagging the C-terminus of NS1A protein were not successful, as no viable virus particles could be recovered from the reverse genetics system. Furthermore, even that such virus can be obtained, it could be much attenuated, and the protein interactions present in these virus-infected cells may not resemble those in wild type non-tagged counterparts. For the same reason, internal tagging of NS1A protein would not be suitable, especially since the structure of the intact NS1A protein is still unknown. Therefore, the N-terminal tagging strategy was employed. Since the N-terminal tag will target both NS1A and NS2A proteins, the purified protein complex will contain both NS1A and NS2A interacting proteins as shown in Figure 3.7.

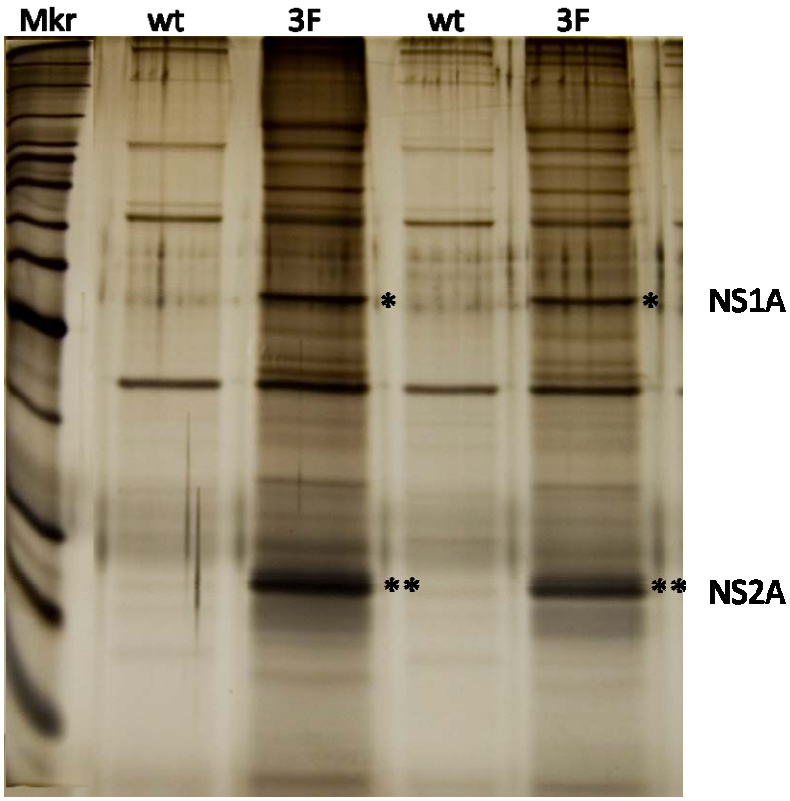


Figure 3.7 **NS2A protein is co-purified with NS1A and other interactors.** A549 cells were infected with either wild-type of N-terminal 3XFlag tagged NS viruses. Peptide eluates after affinity purification were resolved on 12% SDS-PAGE. As indicated, NS1A and NS2A were co-purified in the eluates.

Theoretically, it is still possible to use the virus encoding N-terminal tagged NS proteins to identify NS1A associated factors as proteins identified by mass spectrometry analysis can be validated for its NS1A binding specificity using *in vitro* interaction assay. However, the two-step approach will be time-consuming and lose the advantage the tagging virus strategy provides. Thus, our effort to purify NS1A-specific associated factors had been focusing on the depletion of NS2A from either the lysate or eluates. Two attempts with different strategies were made to achieve this goal: using anti-NS2A serum or including a second different tag on the C-terminus of NS2A protein. As discussed earlier, the drawback of using antiserum is that sometimes it would not provide efficient binding. Two problems had surfaced while using NS2A antiserum-based approach. The NS2A antiserum that we tested could only pull-down a portion of NS2A protein when incubating for several hours as in regular co-immunoprecipitation experiments. When the incubation was prolonged to achieve more efficient NS2A depletion, NS1A was non-specifically pulled down, possibly because the polyclonal antibodies recognize the N-terminus shared by NS1A and NS2A.

The other approach involves the attachment of another epitope tag at the C-terminus of NS2A that differs from the one already presents at the N-terminus of both NS1A and NS2A proteins. Thus, the double-tagged NS2A could be separated from single-tagged NS1A by affinity purification based on the second tag specific to NS2A (Figure 3.8). However, viruses encoding either N-3XFlag-C-2XPy-NS2A or N-2XPy-C-3XFlag-NS2A protein could not be recovered from reverse genetic system. Each genomic RNA segment of influenza A virus contains specific packaging signal encompassing both 5'- and 3'-UTRs and parts of coding sequences. The introduction of two tags on both ends of NS gene may lead to the disruption of packaging signal and, as a result, NS gene segment cannot be effectively incorporated into the virion.

Figure 3.8 Schematic diagram of creating double-tagged NS2A construct. A second epitope tag different from the N-terminal tag was attached to the C-terminus of NS2A protein. As a result, pHH21 vectors containing either N-3XFlag-C-2XPy-NS gene or N-2XPy-C-3XFlag NS gene were generated.

Despite the obstacles listed above, these tagged viruses might still show their worth in elucidating NS1A functions. As described in the introduction, one of the most important NS1A functions is to bind the CPSF 30kD subunit (CPSF30) and thus blocks the maturation of host messenger RNA, including IFN-β mRNA. Our most recent study suggested the existence of a macromolecular complex in infected cells that contains CPSF30, NS1A, and viral polymerases, implicating there might be more profound function of CPSF30-NS1A interaction. In brief, transiently expressed GST-CPSF30 in infected cells can pull down not only NS1A but also viral polymerases and NP protein. To gain further insight into the actual function of this macromolecular complex, it is important to identify its components, and the tagged virus is extremely useful for this purpose. We will use the double purification strategy to isolate the macromolecular complex anchored on CPSF30 and NS1A: GST-CPSF30 expression vector will be transiently transfected into 293T cells which will then be infected with the recombinant viruses encoding tagged NS proteins. It is worth noting that, since GST-CPSF30 only binds to NS1A but not to NS2A, the first purification will eliminate the NS2A interference that has been the major concern for using these tagged viruses. GST-CPSF30-NS1A complex will be first isolated by GST purification and glutathione elution. The eluate will be subjected to the second affinity purification based on the specific epitope tag on NS1A protein. This double purification approach should result in high-purity isolation of NS1A-CPSF30 complex from infected cells, and we would then first determine whether the viral polymerases are indeed the integral part of this complex. Although this experiment will not provide answers to the complete listing of all the NS1A associated proteins in infected cells, since it is limited in only identifying NS1A binding partners in NS1A-CPSF30 complex, it is essential for studying the possible interaction of NS1A with viral polymerases and NP during viral infection. In addition to

viral proteins, host factors, if any, in this macromolecular protein complex will also be specifically identified by the double purification. This will facilitate the research of the functions for this macromolecular protein complex.

Finally, although the recombinant influenza A/Udorn/72 virus encoding N-terminal tagged NS proteins has its limitations in truly identifying NS1A associated proteins during viral infection, it was used for identifying the actual phosphorylation sites on NS1A protein during viral infection as described in the following chapter.

Chapter 4 : NS1A protein of influenza A/Udorn/72 viruses is phosphorylated on both Threonine and Serine residues.

4.1 INTRODUCTION

Phosphorylation is an important post-translational modification that has been shown to regulate various biological activities in the cells. Numerous viral proteins have been shown to be phosphorylated during viral infection. The phosphorylated proteins in influenza A virus include NP (Privalsky and Penhoet 1977; Skorko et al. 1991; Arrese and Portela 1996), NS1(Privalsky and Penhoet 1978; Privalsky and Penhoet 1981; Petri et al. 1982), NS2(Richardson and Akkina 1991), PA(Sanz-Ezquerro et al. 1998; Huarte et al. 2003), M1(Gregoriades et al. 1984; Reinhardt and Wolff 2000), and M2 (Holsinger et al. 1995; Thomas et al. 1998). NS1A phosphorylation was first described 30 years ago using a two-dimensional gel electrophoresis analysis (Privalsky and Penhoet 1978). Infected cells were pulse-labeled with ^{32}P-orthophosphate, and the phosphorylated proteins were examined by two-dimensional gel electrophoresis followed by autoradiography. Two phosphorylated species of NS1A proteins were identified, thin-layer electrophoresis analysis of phosphoamino acids indicated that two threonines (Ts) in the NS1A protein were probably phosphorylated (Privalsky and Penhoet 1981). It was also suggested that the phosphorylation occurred either after or simultaneously with translocation of NS1A into the nucleus. In a later report (Petri et al. 1982), NS1A phosphorylation was reported to vary among strains. Among the viruses tested, only NS1 proteins of human strains are phosphorylated. Ever since then; however, the actual phosphorylation sites on NS1A were never identified, and the biological significance of NS1A phosphorylation was not addressed. In the present study, we identify the

phosphorylation sites of the NS1A protein during viral infection by affinity purification using the influenza A/Udorn/72 virus expressing N-terminal tagged NS1A protein, followed by mass spectrometry, and demonstrate that one of these phosphorylations, at the T at position 215, is critical for virus replication.

4.2 MATERIALS AND METHODS

4.2.1 Cell lines

A549 cells, MDCK cells, , 293T cells, and Hel299 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO®), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO®) at 37°C under a 5% CO_2/95% air atmosphere. Calu3 cells were grown in Advanced MEM (GIBCO®) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under a 5% CO_2/95% air atmosphere.

4.2.2 Generating recombinant influenza A/Udorn/72 viruses by reverse genetics

Recombinant influenza A/Udorn/72 viruses were generated as described in chapter 2. In brief, 293T cells were co-transfected with pHH21 plasmids containing the full-length cDNAs for each of the eight influenza A/Udorn/72 genomic RNA segments, and four pcDNA plasmids expressing NP, PA, PB1, and PB2 proteins of influenza A/Udorn/72 virus. Transfected 293T cells were subsequently overlaid onto MDCK cells for more efficient virus production. Culture supernatants containing recombinant virus particles were collected and used for isolating individual plaques by performing plaque assays in MDCK cells. Serial 10-fold dilutions of viral samples were used for infecting a

monolayer of MDCK cells, and 1% semisolid agarose resuspended in DMEM containing 2.5μg/ml N-acetylated trypsin (Sigma) and antibiotics was overlaid onto the cells. Two to three days after initial infection, individual plaques were picked and stored in 1.2 ml DMEM containing 1% BSA at -80°C. The plaque was used to further amplify the virus in the allantoic cavity of 10-day-old embryonated chicken eggs (Charles River Laboratories) at 34°C. Allantoic fluid was harvested after 48 hours, and virus aliquots were stored at -80°C. Virus titer was measured by plaque assays in MDCK cells. Plaques were visualized by staining with either crystal violet or naphethelene blue-black. Influenza A virus encoding N-terminal 3XFlag tagged NS proteins was generated by inserting the 3XFlag tag sequence between the start codon and the coding sequence of the NS gene in pHH21 plasmid used for 12 plasmids co-transfection. Phosphorylation site-specific mutant viruses were generated by mutating corresponding sites on NS gene in pHH21 plasmids and then used them for co-transfection experiments. Site-specific mutagenesis by PCR was performed to create phosphorylation site-specific mutants by changing threonine to either alanine or glutamic acid. As a result, recombinant influenza A/Udorn/72 viruses encoding NS1A with T49A, T49E, T49Q, T215A, or T215E mutation were generated. Viral genomic RNAs of egg-amplified recombinant viruses were extracted and sequenced to ensure proper introduction of these mutations.

4.2.3 Virus infection and growth curve analysis

Wild-type influenza A/Udorn/72 virus encoding N-terminal 3XFlag tagged NS proteins is referred to as 3Fwt virus. For affinity purification, A549 cells were seeded onto 150mm plates and used for infection when cells reached 95-100% confluency. After washing twice with PBS, A549 cells were either mock-infected or infected with 3Fwt

viruses at a multiplicity of 5 pfu per cell. After an 1 hr adsorption in serum-free DMEM at 37°C, virus inoculum was replaced by DMEM supplemented with 2% heat-inactivated FBS and penicillin-stryptomycin. Infected cells were incubated at 37°C/5%CO_2 for additional 7 hours before harvesting the cell lysates. For multiple cycle growth curve, confluent monolayer of MDCK cells was washed twice with PBS. Influenza virus was diluted with serum-free DMEM and added to the washed cells for virus adsorption at multiplicity of infection (MOI) of 0.001 pfu per cell. After incubation at 37°C for 1h, virus inoculum was replaced by serum-free DMEM supplemented with 2.5μg/ml N-acetylated trypsin (NAT) (Sigma) and penicillin-streptomycin. Supernatants from culture medium containing released viral particles were collected every 12 hours for 60 hours, and virus titer was measured by performing plaque assays in MDCK cells. For single cycle growth curve (SCGC), MDCK cells were infected at MOI of 5 pfu per cell. After 1h virus adsorption at 37°C, cells were washed twice with PBS to remove unbound viral particles before replacing with the infection medium. Supernatants were collected every 2 hour for 10 hours, and virus production was measured by plaque assays in MDCK cells. All experiments were done in triplicates, and virus titers were averaged and used to plot growth curve in logarithmic scale.

4.2.4 Affinity purification and Mass Spectrometry

Eight hours post-infection, mock- or 3Fwt- infected A549 cells were lysed in Tris-buffered saline (TBS; 50mM Tris-Cl, pH 8.0, 150mM NaCl) plus 1% NP-40 supplemented with Complete® protease inhibitor (Roche) and PhoSTOP® phosphatase inhibitor (Roche), followed by sonication with increasing output for 1 minute on ice. Cell lysate was obtained after removing insoluble debris by centrifugation at 14,000rpm for 30

minutes at 4°C. Protein concentration was measured by Bradford assay. Equal amount of protein from each sample was used for affinity purification. Anti-Flag M2 agarose (Sigma) was washed twice with PBS and equilibrated with lysis buffer. Cell lysate was then added to the washed beads and incubated overnight at 4°C for optimal binding. After binding, the beads were extensively washed with lysis buffer and equilibrated with a buffer containing 50mM Tris-Cl, pH7.5, 150mM NaCl, 0.012% Triton X-100, 10% glycerol, 0.2mM EDTA. Elution was then carried out twice using 1:1 (v/v) ratio of M2 agarose and 1μg/μl 3XFlag peptide (Sigma) in the equilibration buffer. Eluates were concentrated using Microcon YM-10 (Millipore). Purified protein was resolved on 12% SDS-PAGE and visualized by colloidal blue staining (Invitrogen).The NS1A protein band was sent to the Taplin Biological Mass Spectrometry Facility at Harvard Medical School for phosphorylation analysis.

4.2.5 Immunofluorescence

Human embryonic lung fibroblasts Hel299 cells were seeded onto 4-well chamber slide. Upon reaching 50% confluency, cells were infected with either wild-type or mutant influenza A virus at MOI of 3 pfu/cell. After 1h adsorption, virus inoculum was replaced by DMEM supplemented with 2% FBS and penicillin-streptomycin. Six hours post-infection, cells were rinsed with ice-cold PBS, fixed by 4% paraformaldehyde in PBS for 20 minutes at room temperature, permeabilized with 0.5% Triton X-100, and blocked with 5% normal goat serum in PBS/0.2% gelatin/ 0.1% Tween-20. Cells were then incubated with rabbit anti-GST-NS1A antibody diluted 1:200 in blocking buffer for 1 hour at room-temperature and washed four times with PBS plus 0.1% Tween-20 (PBST). Fluorescein isothiocyanate-conjugated (FITC) AffiniPure Goat Anti-Rabbit IgG (Jackson

Lab) was diluted 1:100 in blocking buffer, and added to the cells for incubation at room temperature for 1 hour. After washing 3 times with PBST and one time with PBS, the intracellular localization of NS1A protein was visualized using fluorescence microscope.

4.2.6 S^{35} labeling of cells.

A549 cells or Calu3 cells were seeded onto 6-well tissue culture plates. Confluent monolayer cells were either mock-infected or infected with the indicated influenza viruses at a MOI of 5 pfu/cell. At various time points post-infection, cells were washed twice with PBS and replenished with 1ml methionine- and cysteine-free DMEM (GIBCO®). After incubation at 37°C for 15 minutes to deplete unlabeled methionine and cysteine, cells were labeled with 10µl mixture of ^{35}S-L-Methionine and L-Cysteine (TRANS^{35}S™, MP Biomedical) at a final concentration of 0.1mCi/ml for 30 minutes. Cells were then washed twice with ice-cold PBS, and direct lysis was performed with 200µl/well 1X Laemmli SDS sample buffer. Aliquots of the samples were resolved by 12% SDS-PAGE and analyzed by autoradiography.

4.3 RESULTS

4.3.1 Identify phosphorylation sites on NS1A protein of influenza A/Udorn/72 virus using mass spectrometry

Influenza A/Udorn/72 virus encoding N-terminal 3XFlag tagged NS proteins (3Fwt) was generated as described in chapter 3. Previous experiments using this virus to identify NS1A/NS2A associated proteins showed that high stringency washes of affinity columns yielded preparations of NS1A and NS2A proteins with minimal amounts of contaminating proteins. This finding showed that high stringency wash would be useful

in preparing a pure fraction of the N-terminal tagged NS1A protein for identification of the phosphorylation sites on the NS1A protein.

A549 cells were either mock-infected, or infected with 3Fwt influenza A/Udorn/72 virus. After 8 hours of infection, cell lysate was prepared in 1% NP-40 lysis buffer. Because the majority of the NS1A protein is in the nucleus of infected cells, the lysate was sonicated to disrupt the nuclear membrane. 3XFlag tagged NS1A and NS2A proteins were then purified by binding to the anti-Flag M2 agarose, extensively washed with TBS plus 1% Triton X-100, and specifically eluted with concentrated 3XFlag peptide. The eluted proteins were resolved on 12%SDS-PAGE, and the polyacrylamide gel was stained with colloidal blue (Figure 4.1). The protein band corresponding to the NS1A protein was cut out and sent for mass spectrometry analysis.

Mass spectrometry analysis identified 3 sites on the NS1A protein of influenza A/Udorn/72 virus that were phosphorylated during virus infection: S42, T49, and T215. The previous biochemical study of the NS1A protein indicated that it was phosphorylated only on threonine residues, and at two major sites (Privalsky and Penhoet 1981). Our findings of phosphorylation at T215 and T49 probably constitute the two previously reported major phosphorylation sites of the NS1A protein. In addition, our finding of phosphorylation at S42 is the first evidence that serine residues of NS1A protein are phosphorylated during viral infection.

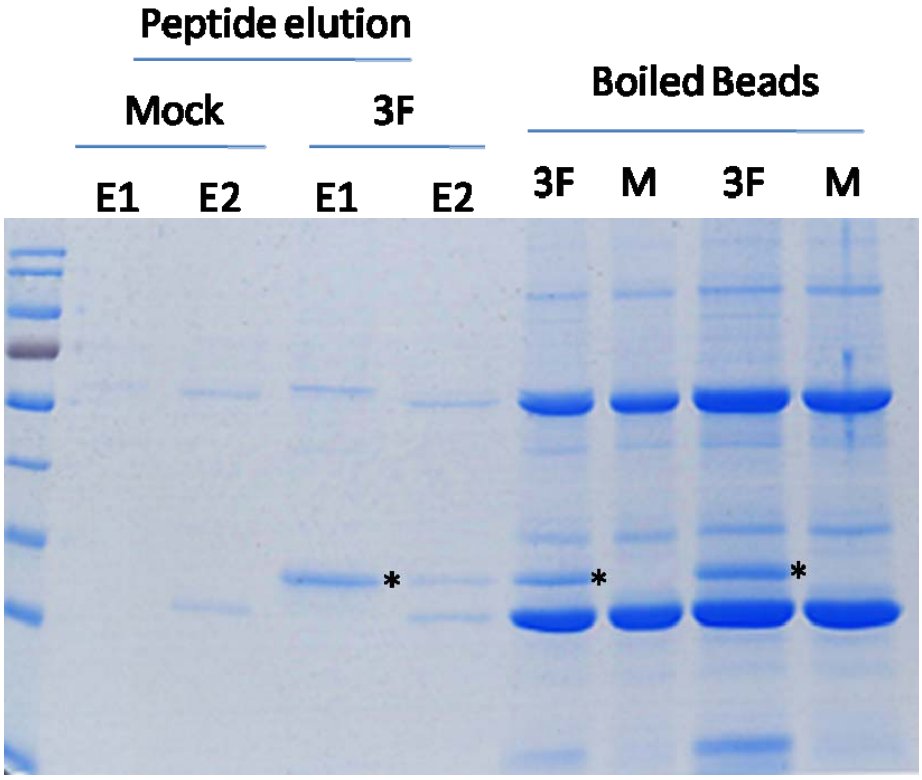


Figure 4.1 Affinity purification of N-3XFlag NS1A protein from infected cell lysate. A549 cells were mock-infected (M) or infected with influenza A/Udorn/72 viruses encoding N-3XFlag NS proteins (3F). After extensive wash and peptide elution, purified proteins were separated on 12% SDS-PAGE. E1 and E2 indicate the first and second elution. * represents the position of N-3XFlag-NS1A.

4.3.2 Generating influenza A/Udorn/72 viruses encoding NS1A with mutated phosphorylation sites

To examine the function of NS1A threonine phosphorylation on viral replication, recombinant mutant influenza A/Udorn/72 viruses encoding NS1A proteins with amino acid substitutions at positions 49 and 215 were generated. By employing PCR site mutagenesis, threonine residue at position 49 or 215 on NS1A was changed to alanine to abolish the phosphorylation; or glutamic acid to mimic constitutively phosphorylated state. Resulting DNAs were cloned into pHH21 vectors and used in the 12-plasmids co-transfection of recombinant system to generate mutant viruses. However, NS2A is a spliced form of NS gene product with +1 frameshift ORF of the coding sequence in its C-terminal exon compared to NS1A. Any change made on NS1A in the overlapping region has to be examined carefully so that the NS2A protein is not affected. For T49 mutants, the change would not affect NS2A protein; however, for T215 mutants, amino acid substitutions in the NS1A protein could affect NS2A coding sequence. In the T215A mutant virus, the amino acid sequence of NS2A was not altered. In the T215E mutant virus, corresponding sequence in NS2A led to a change of leucine to isoleucine, which we considered as a minor alteration and should not affect NS2 function. In fact, as described below, this mutant virus is not attenuated. Genome segments of mutant viruses were sequenced to ensure proper introduction of the mutated sites. As a result, recombinant viruses with NS1A encoding the mutations, T49A, T49E, T49Q, T215A, or T215E, were successfully recovered from supernatant of recombinant system and amplified in embryonated eggs.

4.3.3 Phosphorylation of Thr 49 has negative effect on influenza A/Udorn/72 growth

Recombinant viruses encoding T49A, T49E, or T49Q NS1A proteins were used for plaque assay in MDCK cells. When phosphorylation is abolished by introducing Thr-to-Ala mutation, no obvious difference of plaque sizes were found when compared to wild-type virus. This result would direct us to the conclusion that phosphorylation does not have biological significance at position 49. However, to our surprise, mimicking constant phosphorylated state at position 49 by changing Thr to Glu attenuates the virus, as the mutant virus forms pinpoint plaques on MDCK cells (Figure 4.2). In addition, mutant virus with Thr-to-Asn change at position 49, which resembled the size of phosphorylated Thr without negative charge, was not attenuated in plaque size.

To examine the effect of phosphorylation on viral growth, we compared the viral replication of T49A and T49E mutants to wild-type virus in both single and multiple cycle growth in MDCK cells. For multiple cycle growth curve (Figure 4.3(A)), MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 pfu per cell, and the amount of virus produced was determined by plaque assays. While the T49A mutant virus has almost about the same growth kinetics as wild-type virus, the yield of T49E mutant virus was about one log less than the wild-type virus. The reduction of T49E mutant virus production was also found in single cycle growth curve when MDCK cells were infected at high MOI of 5 pfu per cell (Figure 4.3(B)).

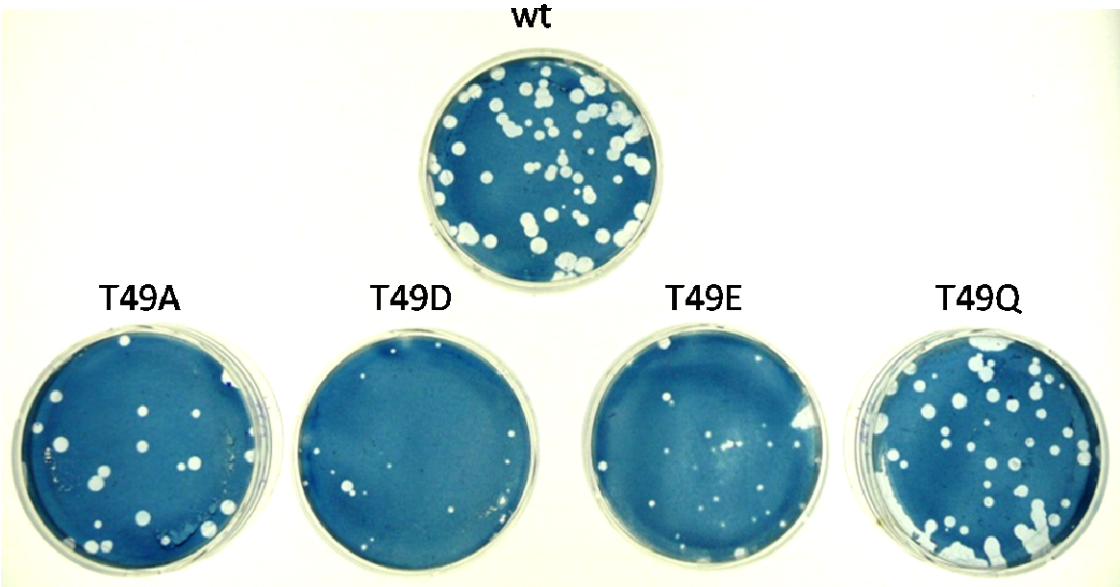
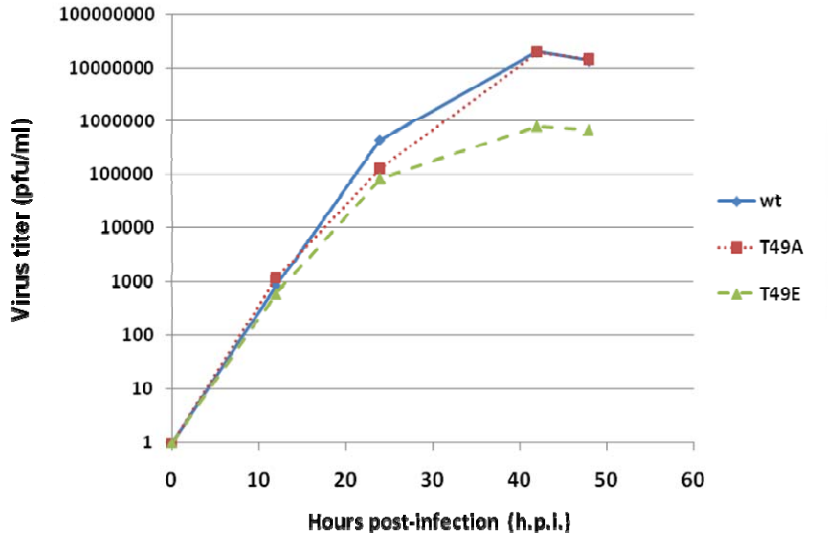


Figure 4.2 Mimicking constant phosphorylated state at position 49 causes virus attenuation. Recombinant influenza A /Udorn/72 viruses encoding Thr-49 mutant NS1A proteins were generated. Thr-to-Ala (T49A) abolished T49 phosphorylation. Thr-to-Asp (T49D) and Thr-to-Glu (T49E) mimicked constant phosphorylated state. Thr-to-Gln (T49Q) imitated the size of threonine with a phosphate group without charge.

(A)



(B)

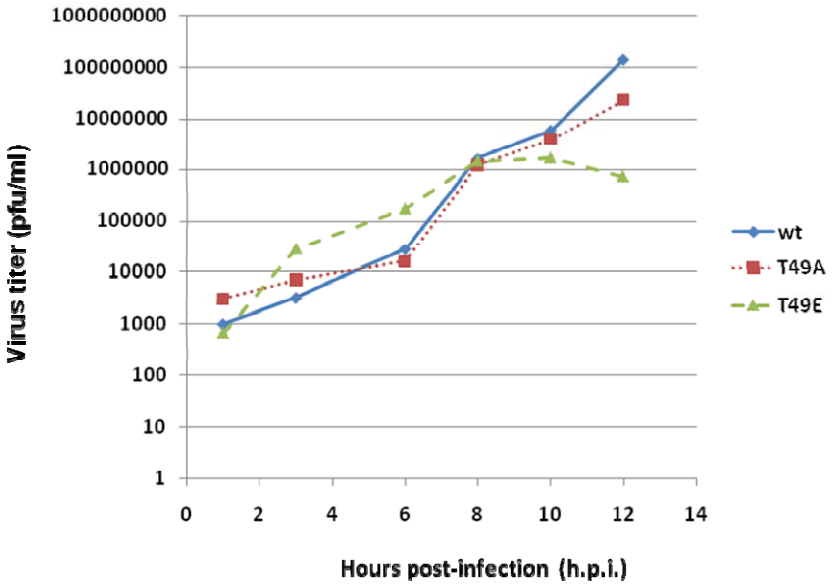


Figure 4.3 Comparison of the viral replication of T49 mutant viruses in MDCK cells. (A) Multiple cycle growth curve. MDCK cells were infected by wild-type (wt) or T49 mutant viruses at MOI of 0.001 pfu/cell. Virus production was measured by plaque assay in MDCK cells at different time points..(B) Single cycle growth curve. MDCK cells were infected with MOI of 5 pfu/cell.

Immunofluorescence showed that the T49A and T49E mutant NS1A proteins, like the wild-type NS1A protein, localized in the nucleus, indicating that the attenuation of the T49E virus was not due to mislocalization of the mutant NS1A proteins (Figure 4.4). These results suggest that phosphorylation at position 49 plays a role in regulating viral growth; however, in a negative aspect.

T49 is highly conserved in human influenza A viruses. If phosphorylation at position 49 had only negative effect on viral growth, one would expect that RNA viruses with high mutation rate like influenza viruses would have changed this site. Therefore, T49 phosphorylation might possess a later positive effect during viral replication, which would not be detected unless phosphorylation was introduced from the beginning as seen in T49E mutant. However, one would also expect that the T49A mutant virus would be attenuated. It is possible that attenuation of T49A was not observed in MDCK cells because of insufficient phosphorylation of T49 in wild-type virus due to insufficient levels of the appropriate kinases.

4.3.4 Phosphorylation of Thr 215 has positive effect on influenza A/Udorn/72 growth

In contrast, mutation of the T215 site shows a different pattern. Recombinant viruses encoding T215A or T215E NS1A proteins were generated as described before. These viruses were used for plaque assays on MDCK cells. When phosphorylation is abolished by introducing a Thr-to-Ala amino acid substitution, the virus formed smaller plaques compared to wild-type virus (Figure 4.5(A)). In contrast, mimicking constant phosphorylated state at position 215 by changing Thr to Glu does not lead to attenuation. No obvious difference of plaque sizes were found when compared T215E to wild-type virus (Figure 4.5(B)). This result indicates that phosphorylation at T215 is important for virus replication.

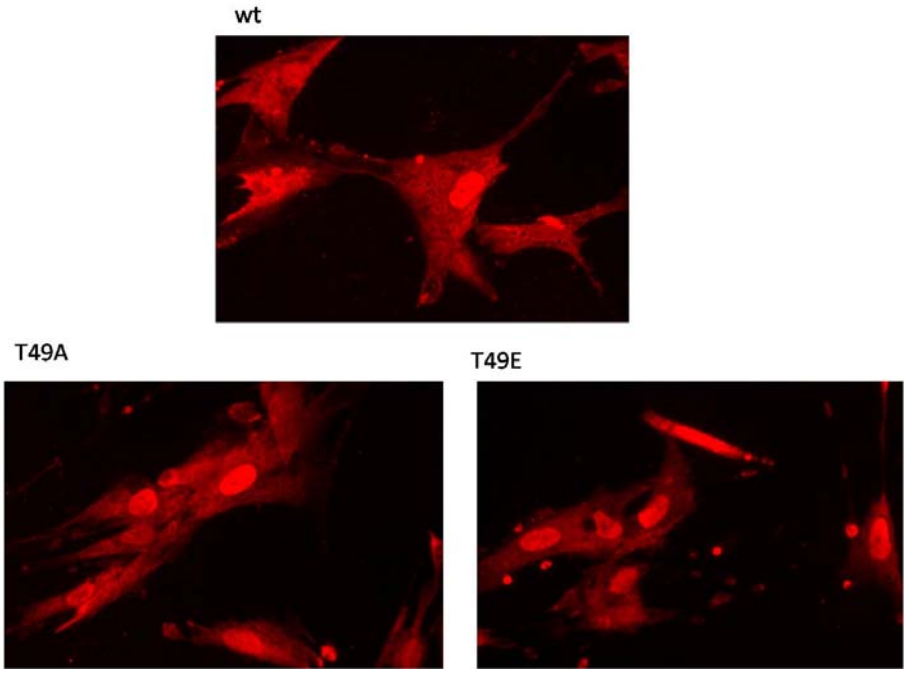


Figure 4.4 T49 mutant NS1A proteins localized in the nucleus. Hel299 cells were infected with wild-type or T49 mutant viruses at MOI of 5 pfu/cell for 6 hours. Localization of NS1A proteins were visualized by immune-fluorescence analysis.

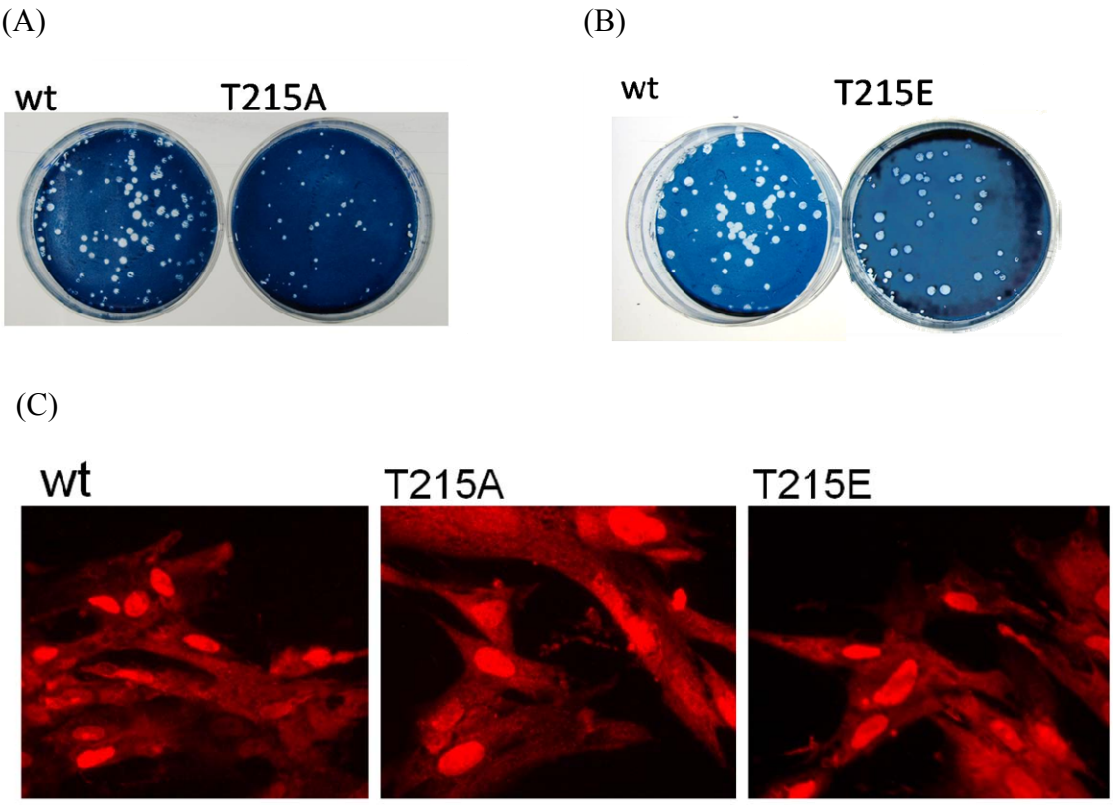


Figure 4.5 Abolishing phosphorylation at position 215 of NS1A protein causes attenuation. Recombinant influenza A /Udorn/72 viruses encoding Thr-215 mutant NS1A proteins were generated. Plaque assay was performed using MDCK cells. Thr-to-Ala (T215A) abolished T215 phosphorylation. Thr-to-Glu (T215E) mimicked constant phosphorylated state. (A) Abolishing T215 phosphorylation results in smaller plaques for T215A mutant virus compared to that of the wild type viral infection. (B) Mimicking constant phosphorylation at T215 (T215E) does not cause virus attenuation. (C) Immunofluoresence. NS1A protein of mutant viruses located at nucleus as seen with wild-type viral infection.

Immunofluorescence showed that theT215A and T215E mutant NS1A proteins, like the wild-type NS1A protein, are localized in the nucleus, indicating that the attenuation of the T215A virus was not due to mislocalization of its NS1A protein (Figure 4.5(C)).

To examine the effect of phosphorylation on viral growth, we compared the rate of viral replication of T215A and T215E mutants to wild-type virus in both single and multiple cycle growth in MDCK cells. For multiple cycle growth curve, MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 pfu per cell, and the amount of virus produced was determined by plaque assays. While T215E mutant showed almost about the same growth kinetics as wild-type virus, the production of T215A mutant virus was about two logs lower than that of the wild-type virus (Figure 4.6(A)). The differences of the viral replication were also seen in single cycle growth curve when MDCK cells were infected at high MOI of 5 pfu per cell. (Figure 4.6(B)). These results show that phosphorylation of T215 plays an important role in the replication of human influenza A viruses.

4.3.5 Abolishing phosphorylation at position 215 of the NS1A protein might change the overall posttranslational modification of the NS1A protein.

^{35}S-methionine and cysteine pulse-labeling was performed to compare the synthesis of virus-specific proteins in cells infected with the T215 mutants and wild-type viruses. Either A549 or Calu3 cells were infected with T215A, T215E or wild-type viruses at MOI of 5 pfu/cell. Cells were pulse-labeled every 2 hours post-infection and analyzed by 12% SDS-PAGE. At each time-point, the majority of virus-specific proteins, including NP, polymerases, and HA, showed no significant difference in protein expression level between wild type virus and the two T215 mutant viruses (Figure 4.7).

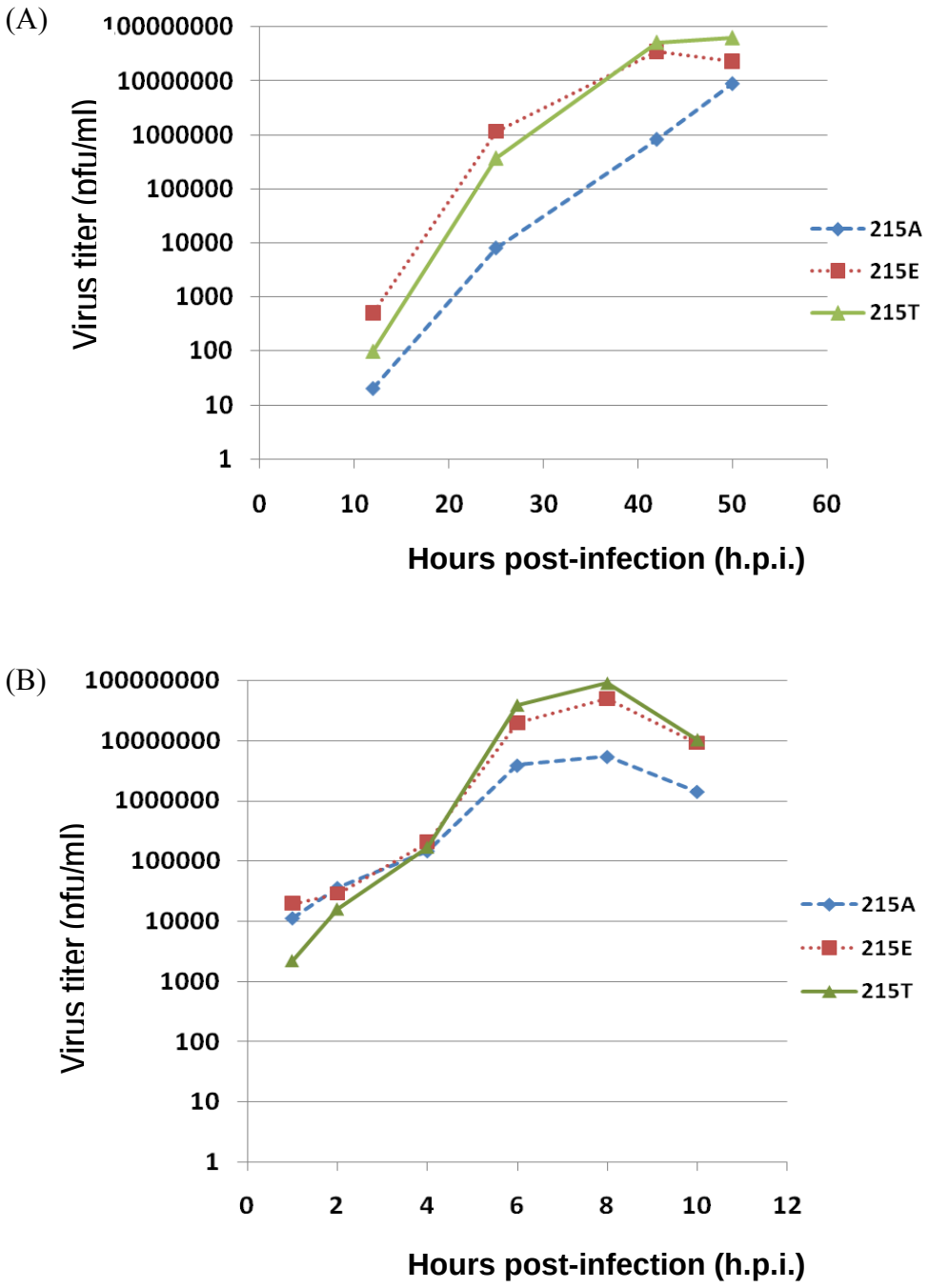


Figure 4.6 Abolishing T215 phosphorylation of NS1A protein causes virus attenuation. (A) Multiple cycle growth curve. MDCK cells were infected at MOI of 0.001 pfu/cell. Virus production was measured by performing plaque assays in MDCK cells. (B) Single cycle growth curve. MDCK cells were infected at MOI of 5 pfu/cell.

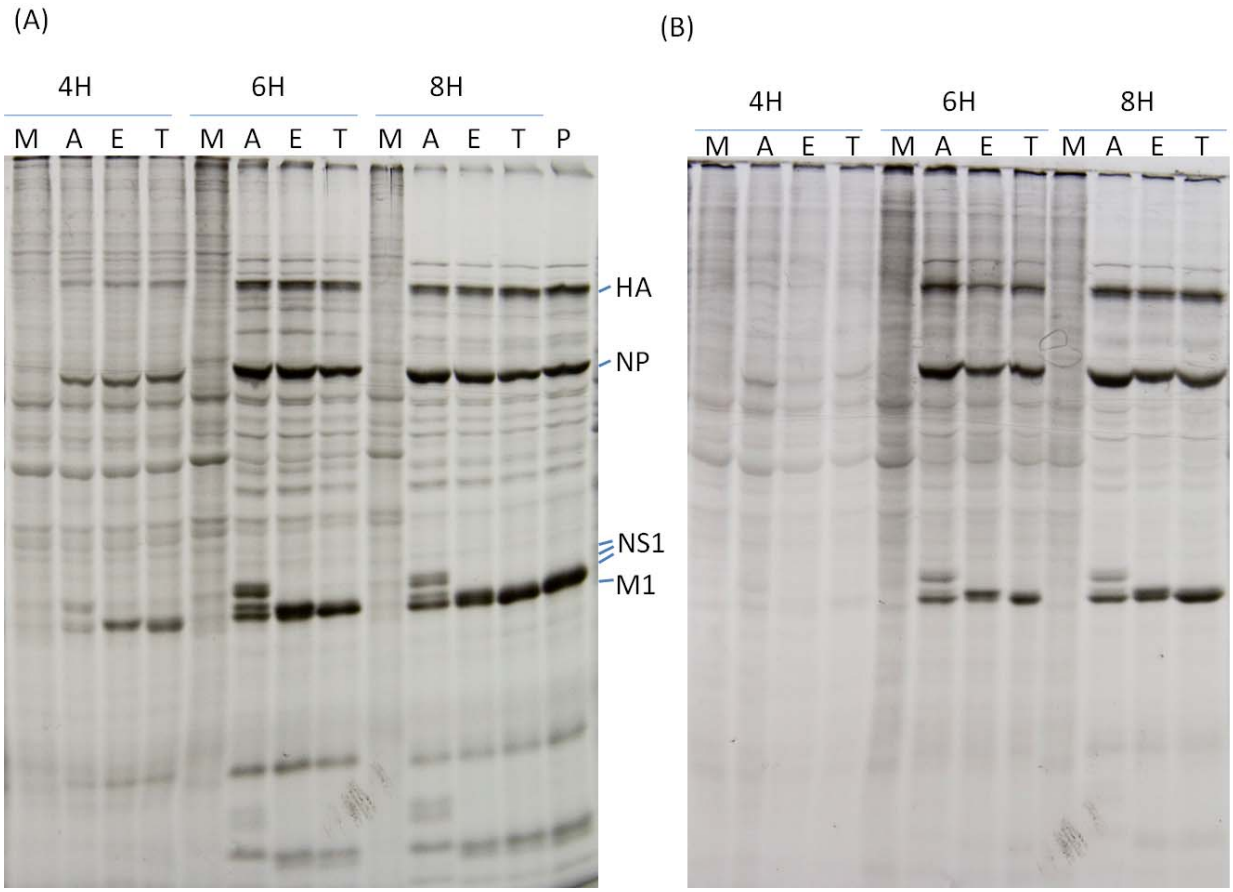


Figure 4.7 Abolishing T215 phosphorylation at position 215 might change the overall posttranslational modification of NS1A protein. Cells were either mock-infected (M) or infected with wild-type (T), T215A (A), or T215E(E) influenza A/Udorn/72 viruses at MOI of 5 pfu/cell. ^{35}S pulse-labeling were carried out at time-points indicated. (A) A549 cells. (B) Calu3 cells.

However, in cells infected with the T215A mutant virus, four newly synthesized proteins, in comparison to two in samples from wild type virus and T215E virus-infected cells, were observed in the SDS-PAGE gel close to the region where NS1A and M1 proteins migrate (Figure 4.7). Further Western blot analysis demonstrate that the protein with the greatest mobility is the M1 protein, and the other three bands with lower mobility are NS1A proteins. The combined level of synthesis of these three NS1A species is comparable to that of the single species of the NS1A protein synthesized in cells infected by either the T215E mutant or wild-type viruses.

These mobility shifts of the NS1A proteins in SDS-PAGE are most likely caused by an overall change in post-translational modification when phosphorylation at T215 is abolished. Decreased mobility of these NS1A protein species suggests that they may have acquired other protein modifications, probably phosphorylations, in addition to the T215 phosphorylation. The introduction of new NS1A post-translational modifications by the T215A mutation might be either directly due to the mutation at position 215 exposes the normally hidden protein surface for additional modification or indirectly resulted from the alteration of interaction of NS1A with cellular proteins. We hypothesize that these changes in the NS1A protein lead to the inhibition of specific NS1A functions, causing the attenuation of the T215A virus (see Discussion).

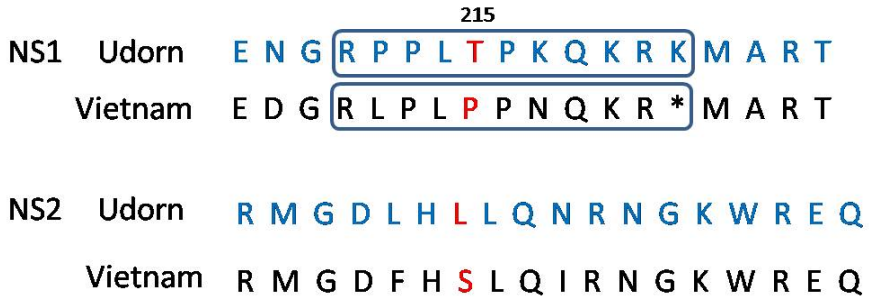
4.3.6 Generating recombinant A/Udorn/72 virus encoding NS1A with Thr-to-Pro mutation at position 215

The identity of the amino acid at position 215 of the NS1A protein discriminates human influenza viruses from avian influenza viruses (Finkelstein et al. 2007), as threonine at this position is conserved in all human strains, whereas proline is present at this position in most avian strains. It is possible that the phosphorylation of Thr-215

confers NS1A function(s) similar to that already exist in avian NS1A with Pro-215. To examine this possibility, a recombinant influenza A/Udorn/72 virus encoding T215P NS1A protein was generated. Due to the necessary codon change, a single amino acid change occurs in the NS2 protein. For this reason, we introduced the Thr-to-Pro mutation into the Udorn NS1A protein, but that the amino acid of NS2A is the same as that of NS2A of avian influenza A viruses (Leu to Ser) (Figure 4.8(A)). Thr-to-Pro recombinant mutant virus was generated as described above. As shown in Figure 4.8(B), the plaque size of T215P mutant is the same as that of wild-type virus, indicating that when threonine 215 was changed to proline, the virus was not attenuated. This conclusion was further confirmed by measurement of viral growth in MDCK cell. No significant difference was detected between T215P mutant, T215E mutant and wild-type viruses in both single- and multiple- cycle growth analysis (Figure 4.9).

The ^{35}S pulse-labeling experiments revealed that NS1A protein of the T215P mutant does not exhibit the SDS page mobility shift observed for the NS1A protein of the T215A mutant (Figure 4.10). We thus conclude that the phosphorylation at Thr-215 in human strains can be functionally replaced by proline residue that is specific for avian strains. One possible explanation for this functional equivalence is that the phosphothreonine at position 215 enables the NS1A protein of human influenza virus strains to bind to the same protein partner(s) as avian strains via their Pro-215-containing sequence.

(A)



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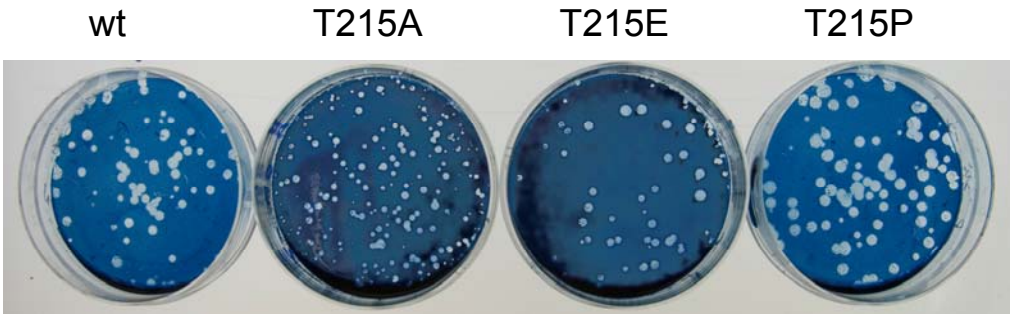
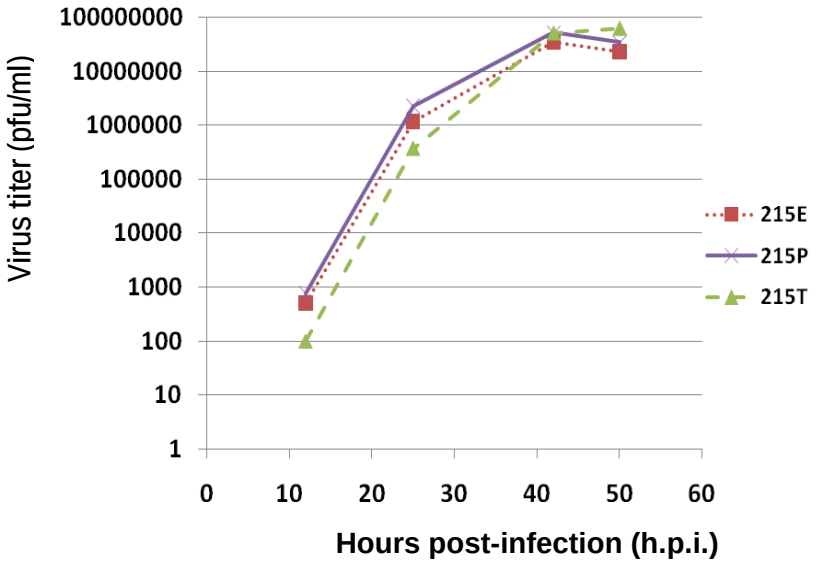


Figure 4.8 Influenza A virus encoding Thr-to-Pro mutation at position 215 has the same phenotype as wild-type virus. (A) Schematic diagram comparing T215 regions of NS1A proteins of human and avian influenza viruses.T215P NS1A influenza A/Udorn/72 mutant virus was generated with Leu to Ser mutation in the corresponding region of NS2A proteins to resemble the region of avian strain. (B) Plaque size comparison of wild-type, T215A, T215E, and T215P NS1A influenza A/Udorn/72 viruses in MDCK cells.

(A)



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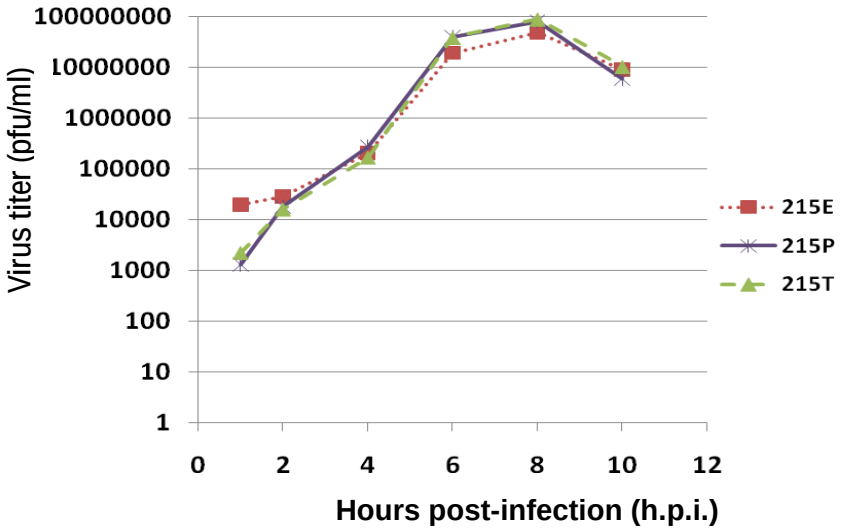


Figure 4.9 Thr-to-Pro mutation at position 215 of NS1A protein of human influenza A/Udorn/72 virus does not affect viral replication. (A) Multiple cycle growth curve. MDCK cells were infected at MOI of 0.001 pfu/cell. Virus production was measured by plaque assays in MDCK cells. (B) Single cycle growth curve. MDCK cells were infected at MOI of 5 pfu/cell.

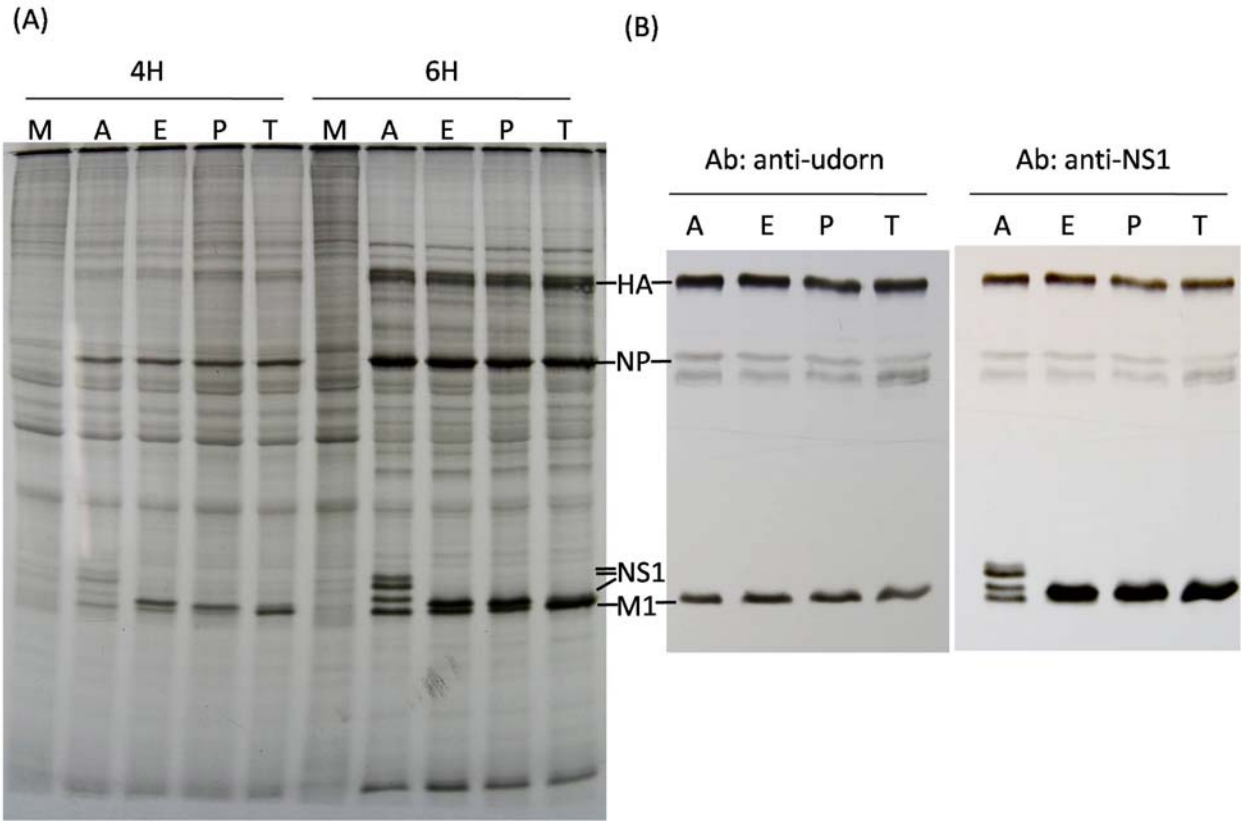


Figure 4.10 Abolishing phosphorylation at position 215 changes the mobility of NS1A protein on 12% SDS-PAGE. A549 cells were either mock-infected or infected by wild-type (T), T215A (A), T215E (E), or T215P (P) NS1A mutant influenza A/Udorn/72 viruses at MOI of 5 pfu/cell or mock-infected (M). ^{35}S pulse-labeling were carried out at time points indicated. (A) Labeled infection cell lysates were resolved on 12% SDS-PAGE and documented by radioautography. (B) Western blot analysis of radio-labeled cell lysates. Blot was first probed with goat anti-udorn antibody to detect HA, NP, and M1. The same blot was used subsequently for detecting NS1A by using rabbit anti-NS1A antibody.

4.4 DISCUSSION

It has been 30 years since the first report of that the NS1A protein contains phosphorylated threonine residues (Privalsky and Penhoet 1981), and the actual sites and biological functions of NS1A phosphorylation remained elusive. Here, by developing a recombinant influenza A virus with N-terminal epitope-tagged NS gene, we were able to purify NS1A protein from infected cell to homogenicity for mass spectrometry analysis which identified the sites of the NS1A protein that are phosphorylated during viral infection. Specifically, our analysis revealed that NS1A protein is phosphorylated at T49 and T215. It is likely that these sites comprise the two threonine phosphorylations suggested 30 years ago. In addition, we discovered a serine phosphorylation site (at Ser-42 of NS1A protein that was not detected previously).

The focus of the present study was on the two threonine phosphorylation sites of NS1A protein. In order to study the effects of NS1A phosphorylation on viral replication, we generated recombinant mutant viruses targeting the phosphorylation sites, which either contain Thr-to-Ala replacement to abolish phosphorylation, or Thr-to-Glu to mimic a constitutive phosphorylated state. The comparison of these mutant viruses with wild type virus in viral growth demonstrated that the phosphorylation of NS1A protein at different threonine residues may impact its function differently: phosphorylation of Thr-215 position is important to support viral growth, while Thr-49 phosphorylation had negative effects on viral replication.

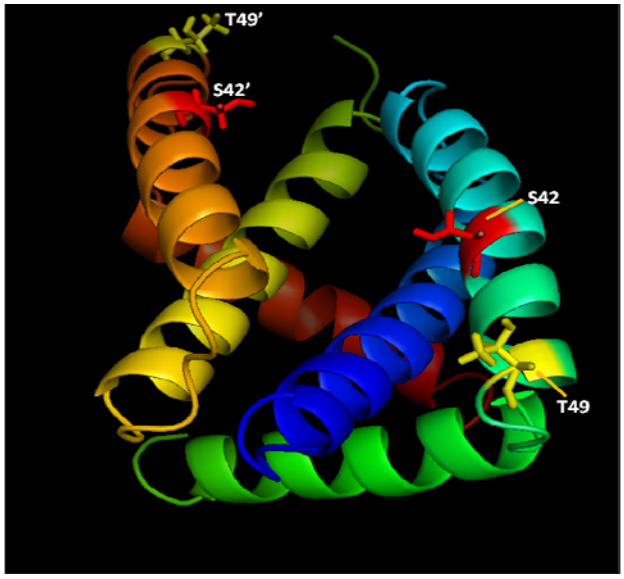
The behavior of the mutations at Thr-49 was surprising. When phosphorylation was abolished by changing Thr to Ala, the mutant virus resembles wild-type viruses. On the other hand, when constitutive phosphorylation was mimicked via the Thr-to-Glu mutant, the virus is attenuated. A mutant virus encoding NS1A with a change of threonine to glutamine (Thr-to-Gln), which mimicked the size of threonine plus a

phosphate group without the charge, behaves similar to wild-type virus, indicating the attenuation seen in T49E virus results from the charged side chain. The attenuation of T49E mutant virus indicates that phosphorylation at this position inhibits virus replication. However, if this is the case, we might expect that the Thr-to-Ala mutation would enhance virus replication, which was not observed. One explanation for this discrepancy would be the kinase that phosphorylates T49 is deficient in MDCK cells, and thus NS1A protein of wild-type virus is not efficiently phosphorylated at T49 position. So the differences of viral replication between T49A and wild-type influenza viruses are not obvious. Concomitantly with searching for the kinase responsible for T49 phosphorylation, we are currently comparing the growth of T49A mutant virus and wild type virus in different cell lines which are commonly used in studying influenza A viruses.

Phosphorylation is arguably one of the most important post-translational modifications, and numerous cellular kinases participate in regulating multiple vital cellular processes. Inevitably, some of the viral encoded proteins, like many of the cellular proteins, undergo phosphorylation as first indicated by their possession of consensus site(s) for specific kinase. Although in some cases the phosphorylations of viral proteins do not affect their functions and are not essential, e.g. the phosphorylation of M2 protein in influenza A virus (Thomas et al. 1998), most of the time viruses take advantage of these modifications to benefit their own replications.

Thr-49, which is highly conserved among influenza A viruses, is located at the end of helix 2 of NS1A RNA binding domain in proximity to the dsRNA-binding groove (Figure 4.11). The most reasonable explanation for the attenuation of NS1A T49E mutant virus is that the substitution of T49 by negatively charged Glu residue abolishes or decreases the dsRNA binding activity of NS1A protein. Consequently, the 2'-5'-OAS

(A)



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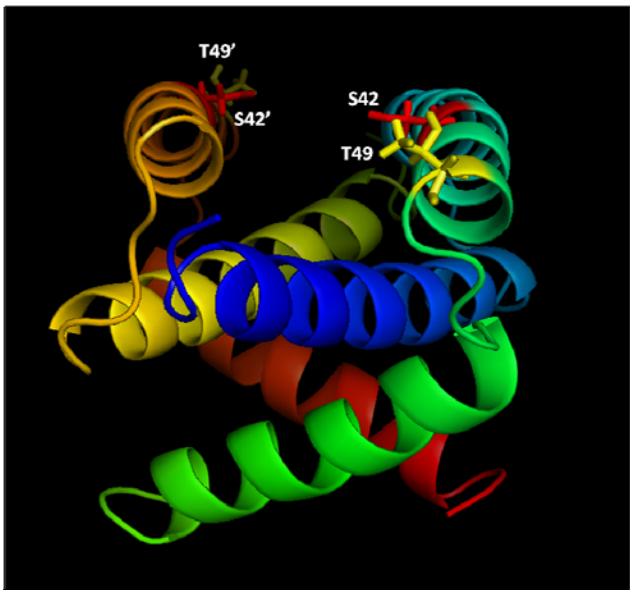

Figure 4.11 Schematic diagram of phosphorylation sites on the NS1A RNA-binding domain. Phosphorylation sites of NS1A RNA-binding domain, Ser42 and Thr49, which are identified by mass spectrometry analysis, are shown as indicated on each monomer of the dimeric structure. (A) Top view (B) Front view.

pathway is reactivated in cells infected with T49E mutant virus, which is similar to the R38A mutant virus (Min and Krug 2006), and it inhibits the viral replication. To validate this theory, our future experiment will first measure the dsRNA binding activity of T49E NS1A protein *in vitro* using recombinant proteins. In parallel, we will compare the growth of T49A and T49E mutant viruses in RNase+/+ and RNase L-/- MEFs to determine their abilities in inhibition of 2'-5'-OAS activation *in vivo*.

If the constitutive phosphorylation of Thr-49 of NS1A protein, as mimicked by T49E mutation, negatively regulates viral replication, why does the highly mutable influenza A virus still retain the Thr-49 residue? We propose that the phosphorylation of Thr-49 of NS1A protein might play a regulatory role in the process of apoptosis induced by influenza viral infection. Influenza viruses induce apoptosis in various cell types both *in vivo* and *in vitro* (Schultz-Cherry et al. 2001; Takizawa et al. 1993; Hinshaw et al. 1994; Zhirnov et al. 2002; Brydon et al. 2005; Zhirnov and Klenk 2007; Lam et al. 2008) and NS1A is identified as one of the viral factors controlling this apoptotic process. An interesting notion has emerged from the studies that the regulation of NS1A on the apoptosis in infected cells could occur at different stages with different outcomes (Weisan Chen et al. 2001; Zhirnov et al. 2002; Bruns et al. 2007). Recently a number of studies have reported that NS1A interacts with p85β subunit of PI3K kinase, leading to the activation of Akt kinase and the down-stream anti-apoptotic signaling pathway (Ehrhardt et al. 2007a; Ehrhardt et al. 2007b; Ehrhardt et al. 2006; Shin et al. 2007b; Shin et al. 2007a; Hale et al. 2006; Li et al. 2008; Hale et al. 2008; Shin et al. 2007). This anti-apoptotic cellular status, which is present in the early stage of infection as indicated by transient activation of the Akt kinase (Zhirnov and Klenk 2007) may provide viruses a window for efficient replication in infected cells. During the late stage of infection, when the viral replication is almost complete, pro-apoptotic signals take

over, and the cellular status shifts from anti-apoptotic to apoptotic. This shift is important for influenza virus propagation. Indeed, caspase 3 activation is critical for the migration of the viral RNPs from the nucleus to the cytoplasm in the infected cells during late infection (Wurzer et al. 2003). Our preliminary data has revealed that this fine-tuned balance between anti-apoptotic and pro-apoptotic statuses is deregulated in cells infected with T49E mutant viruses. Compared to T49A mutant or wild-type virus, T49E mutant virus induces caspase 3 activation at very early stage of infection. This early induction of apoptosis in infected cells is probably responsible for the attenuated phenotypes of the T49E mutant virus. How does the T49E mutation of NS1A protein lead to early apoptosis in infected cells? The answer may lie in the 2'-5'-OAS pathway which is inhibited by wild-type NS1A protein during infection. It is known that activation of RNase L by 2'-5'-OAS leads to release of cytochrome c into the cytosol and caspase activation (Rusch et al. 2004). Thus, the activation of 2'-5'-OAS pathway in cells infected with NS1A T49E mutant may render cells more prone to apoptosis in the early stage of infection. Together, the above listed evidences and rationales lead us to hypothesize that the Thr-49 phosphorylation of NS1A protein occurs at late stage of infection and regulates the apoptotic event. The phosphorylation of Thr-49 might disrupt the dsRNA binding activity of NS1A protein and relieve the inhibitory effect of NS1A on 2'-5'-OAS. To prove this hypothesis, we need to first develop an anti-NS1A antibody specific for phospho-threonine at position 49, which will enable us to determine the time course of Thr-49 phoshorylation during infection. In addition, we need to identify the kinase responsible for the Thr-49 phosphorylation. Intriguingly, our initial computational analysis by using web-based phosphokinase prediction softwares, such as NetPho, indicates that Akt kinase, which mediates anti-apoptotic signaling as discussed above, is the best kinase candidate for Thr-49 phosphorylation. Thus we will examine the candidacy of Akt kinase

using *in vitro* kinase assay in which both the T49 mutant and wild-type NS1A proteins will be used as substrates.

It is intriguing that T215 is one of the phosphorylation sites identified, because this amino acid is a signature for human influenza A virus strains. All human influenza A viruses contain threonine at this position in the NS1A protein, whereas avian influenza A viruses contain proline at this position. A recent report has shown that by retaining the avian P215 signature, the tail regions of NS1A proteins of the 1918 virus and H5N1 viruses form a SH3-domain, which can bind to Crk/CrkL proteins (Finkelstein et al. 2007). This result suggested that the sequence surrounding position 215 might be important for protein-protein interactions. In present study, elimination of T215 phosphorylation by replacing Thr with Ala resulted in decreased virus replication in both multiple cycle and single cycle growth. In contrast, no attenuation occurred when Thr was changed to Glu to mimic constitutive phosphorylation. The latter observation indicates that either most of the NS1A proteins are phosphorylated at position 215 during infection, or the NS1A protein only need a threshold of T215 phosphorylated species to exert the related biological functions. Although the T215A mutant virus is attenuated in virus production, the rate of viral protein synthesis in T215A virus-infected cells is similar to that in cells infected by the T215E mutant and wild type viruses. However, the NS1A protein in T215A virus-infected cells does not migrate as a relatively homogeneous species, as is the case for the wild-type and T215E mutant protein, but rather migrates as three slower moving species. These protein mobility shifts might be the causes underlying the attenuation of virus growth observed. The homogeneous wild-type and T215E species corresponds to the NS1A protein containing the S42, T49 and T215 phosphorylations. One of the slower migrating NS1A proteins in T215A mutant virus infected cells is presumably the NS1A protein lacking phosphorylation at T215.

The other 2 species may represent NS1A proteins containing additional post-translational modifications besides T49 and S42 phosphorylation. These novel post-translational modifications could arise from either the alteration of protein-protein interaction or structural changes, which are less likely, associated with the T215A mutation. One of our immediate future focuses is to purify all three species of NS1A T215A proteins from infected cells using tagged virus approach as described above and identify the post-translational modification associated with each species by mass spectrometry analysis. Such identification may provide first mechanistic insight into how T215A mutation compromises the NS1A function during infection. As described above, in order to analyze the effect of phosphorylation on modified protein, it is important to identify the responsible kinase and this would be one direction for the future study of T215 phosphorylation. Our initial bioinformatics analysis predicted multiple kinases as potential candidates for catalyzing T215 phosphorylation. These candidates include Proline-dependent S/T kinase Cdc2 and Cdk5, GSK3, and MAPK. We will first use *in vitro* kinase assay to examine the candidacy of these kinases using both wild type NS1A and T215A mutant as substrates. Along with the above future directions, we will take a shortcut approach to investigate whether the T215 phosphorylation affects the interaction of NS1A and cellular proteins. This approach is inspired by the finding that the replacement of T215 with Pro is not attenuating. This substitution makes the C-terminal tail of mutated Udorn NS1A protein resemble that of the NS1A proteins of avian origin. Avian NS1A proteins with proline at position 215 form a SH3 domain (P-X-X-P), which has been reported to bind to the Crk and CrkL proteins (Heikkinen et al. 2008). This interaction between NS1A and CrkL appears to enhance PI3K induced Akt signaling, as it has been suggested that CrkL functionally interacts with PI3K complex. It is conceivable that phosphorylated of T215 in the wild-type human (Ud) NS1A protein

provides a novel motif that binds cellular proteins with functions that are similar to those bound by the SH3 domain of avian NS1A proteins. Alternatively, phosphorylation of T215 may achieve the same function as the avian SH3 domain by a totally different mechanism. We are currently starting to investigate this possibility by examining the interaction between Crk/CrkL and NS1A proteins from T215E, T215P, T215A mutant or wild-type virus infected cell lysates.

The present study revealed that the attenuation of T215A mutant virus is caused by defects after viral protein synthesis, most possibly at one or more steps in process of virus particle production. It reminds us a recent study on Hepatitis C virus (HCV) which established that the elimination of a serine phosphorylation of HCV NS5A protein inhibits virus replication at the step of virus assembly without affecting early RNA replication steps. (Tellinghuisen et al. 2008). It is interesting to note that the HCV NS5A protein shows striking similarity to the influenza A virus NS1A protein in several other respects. Not only is NS5A a RNA-binding protein (Huang et al. 2005), it is also an important viral factor in subverting the IFN response in HCV infected cells. NS5A interacts with PKR (Gale et al. 1998) and 2'-5'-OAS (Taguchi et al. 2004) to repress cellular antiviral activity. It promotes PI3K/Akt anti-apoptotic pathway by interacting with the SH3 domain of the PI3K p85 regulatory subunit (Street et al. 2004). NS5A also binds viral encoded RNA-dependent RNA polymerase NS5B and modulate its activity (Shirota et al. 2002). Thus, the extensively studied phosphorylation of HCV NS5A protein may provide a model for the future studies of NS1A phosphorylation of influenza A virus. Further studies of the activities and the regulation of these virus-encoded multifunctional nonstructural proteins will shed new light on viral pathogenesis and ultimately lead to new therapeutics with less side effect and improved efficacy.

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